BIOREMEDIATION OF DIESEL CONTAMINATED SOIL USING BIOSTIMULATION, BIOAUGMENTATION AND BULKING AGENTS

AYOBAMIDELE PHILIP, AKINNOLA

BIOREMEDIATION OF DIESEL CONTAMINATED SOIL USING BIOSTIMULATION, BIOAUGMENTATION AND BULKING AGENTS

By

Ayobamidele Philip, Akinnola
B.Sc. (Biochemistry)

A Thesis Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree of

Master of Engineering

Faculty of Engineering & Applied Science

Memorial University of Newfoundland

August 2005

St. John's Newfoundland Canada

Your file Votre référence
ISBN: 978-0-494-19412-6
Our file Notre référence
ISBN: 978-0-494-19412-6

ABSTRACT

Petroleum hydrocarbons account for approximately 60% of contaminated sites in Canada. Atlantic Canada, especially in the Eastern region of the Province of Newfoundland and Labrador, which is known for quite a number of hydrocarbon-contaminated soils.

Laboratory experiments in two phases were undertaken to compare the influence of nutrients, inocula and bulking agents additions on the bioremediation of diesel-fuel contaminated soil over a 90-day testing period. Phase I experiments determined the effect of one type of nutrient (either poultry manure or liquid cow manure), one type of inoculum (either indigenous or exogenous microbial inoculum) and one type of bulking agent (either sand or hay) on the degradation of diesel fuel in soil. Phase II experiments involved a series of laboratory-based experiments conducted to study the interactions among the nutrients, inocula and bulking agents additions.

After a 90-day experimental period, 96.6% degradation was achieved in contaminated soil containing clean Ottawa sand as a bulking agent in phase I experiments while 96.2% degradation was achieved in contaminated soil containing an inoculum of soil indigenous microbes and clean Ottawa sand in phase II experiments. The biodegradation results were analyzed to determine the most significant factors and interactions using Design-Expert® version 6 software for Design of Experiments. Additions of nutrients and bulking agents was found to be statistically significant, while the addition of inocula and the interactions among the nutrients, inocula and bulking agents were statistically significant.

ACKNOWLEDGEMENTS

I gratefully acknowledge the financial support provided by Faculty of Engineering and Applied Science at Memorial University of Newfoundland and Dr. Cynthia Coles's grant which made this work possible.

I am grateful to my research supervisor, Dr. Cynthia Coles for her gentle nature, guidance, encouragement and active support through the course of this work. I have been fortunate to be advised by Dr. Cynthia Coles and to change my master's degree program into the area of Geoenvironmental Engineering, which will be of immense benefit and of valuable knowledge in my career.

My special thanks to my co-supervisor Dr. Takor Patel from the Department of Biology and to Dr. Robert J. Helleur from the Department of Chemistry at Memorial University of Newfoundland for their close attention and expert and invaluable support in the biological experiments and chemical analysis of this research study. I am grateful to Adewale Lambo, Linda Windsor, Sandra Estevez and Samuel Mugo, for their technical expertise. I acknowledge the personnel of the four laboratories involved in this work: Faculty of Engineering-MUN; Biology Department-MUN; C-CART, Chemistry Department-MUN and Maxxam Analytics Inc. St. John's, Newfoundland.

I say a big thank you to my wife-Funmilola and to my dad and siblings- Paul, Funmilayo and Wale for the love, understanding and support given to me.

Lastly, I am grateful to the Most High God for his inexplicable flow of grace and mercy so far in my life. I would not have come this far without Him.

AYOBAMIDELE

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

atm	Atmosphere
°C	Centigrade
°	Degree
#	Number
%	Percentage
K_d	Absorption coefficient
BOD	Biological oxygen demand
K_{oc}	Carbon Matter Partition Coefficient
C:N:P	Carbon: Nitrogen: Phosphorus
cm	Centimeter
cfu/g	Colony forming units
DF	Diesel fuel
DCM	Dichloromethane
FID	Flame ionization detector
GC	Gas chromatography
g	Gram
hr	Hour
ID	Internal diameter
Kg	Kilogram
l	Litre
MSD	Mass selective detector
MS	Mass spectrometry

mp	Melting point
m^3	Meter cube
Hg	Mercury
µl	Microliter
ml	Mililitre
Mg	Milligram
mm	Millimeter
MSS	Mineral salt solution
min	Minutes
mol	Mole
nm	Nanometer
NAPL	Non-aqueous phase liquids
K_{ow}	Octanol/water partition coefficient
OD	Optical Density
ppm	Parts per million
PHC	Petroleum hydrocarbons
Ibs	Pounds
psi	Pounds per square inch
PAHs	Polycyclic Aromatic Hydrocarbons
rpm	Revolutions per minute
s	Second
TPH	Total Petroleum Hydrocarbon
TSA	Trypticase Soya Agar

UV	Ultraviolet
USTs	Underground storage tanks
v/v	Volume per volume
S_w	Water Solubility
w/v	Weight per volume

Chapter 1

Introduction

1.1 Petroleum Contaminated Sites

A naturally occurring liquid that originates from liquid fossil fuels is termed crude oil, or more appropriately petroleum. Petroleum is of a variety of compositions and complexity and has become the world's foremost source of energy and the essential foundation of many industrialized communities. It has been widely reported that petroleum accounts for 38% energy usage worldwide (Katherine, 2001). While the importance of petroleum as a source of energy is renowned, so also is the environmental pollution that occurs as a result of its widespread usage. Thus, hydrocarbon pollution of the natural environment has been an important issue in recent years. Soil and groundwater contamination by petroleum products is of major concern and the contamination of the environment by petroleum hydrocarbons is both widespread and frequent.

A petroleum-contaminated site refers to any land, surface water and groundwater area upon which petroleum or petroleum products exit at levels which pose existing or imminent threats to human health or the environment. Petroleum hydrocarbons account for approximately 60% of contaminated sites in Canada, which have resulted in a number of problems, such as fire/explosion hazard, human and environmental toxicity, movement through soil to air or water, odour, and impairment of soil processes such as water retention and nutrient cycling (CCME, 2003).

The Eastern region of the Province of Newfoundland and Labrador in Atlantic Canada; a region defined as the area east and south of Shoal Harbour and Swift Current, including the entire Avalon Peninsula, is known for quite a number of hydrocarbon contaminated sites, that must be remediated (Environment Canada, 2003). These include:

- **United States Naval Facility, Argentia, NL**: It has reported that there are approximately 60 localized sites on the Argentia base contaminated with petroleum hydrocarbons, localized PCBs and heavy metals. Leachate containing PCBs and other contaminants has been identified entering Placentia Bay from one landfill.

- **Former Mt. Harmon US Airforce Base, Stephenville, NF:** This site is presently undergoing a site assessment by Environment Canada. Site assessments have also been conducted by Transport Canada and the Newfoundland Labrador Department of Housing.

Hence, the clean up of contaminated sites in Atlantic Canada is important to protect people and the environment.

When petroleum hydrocarbons are discharged to the environment, site restoration may be required to contain the impact of soil and groundwater contamination. Most of the time, remediation is required for petroleum contaminated sites restoration and many different technologies have been developed: biological treatment, soil washing with surfactants, air stripping, thermal desorption, incineration etc. (Kostechi and Calabrese, 1989). However, a comprehensive site assessment is necessary to evaluate the impact of petroleum contamination on the environment and on human health before a remediation process can be selected and implemented, as this would provide much needed framework to make rational and defensive decisions about remedial actions. Once the extent of a petroleum contamination zone is recognized, a remediation strategy needs to be developed using the information derived from a thorough site-characterization and hydrogeological evaluation (Rosenbaum-Wilkinson, 1994).

The hydrogeological evaluation of a petroleum-contaminated site provides a description of the hydrology, geology and geochemistry of the unsaturated and saturated zones. Site-characterization involves the description of the soil classification and morphology; soil physical, chemical and biological properties in the vadose zone and saturated zones; and geological interpretation of subsurface sediment and rock structure, layering, depth and fractures. Site-characterization also involves the estimation of changes in hydraulic conductivity with depth, the seasonal variations in precipitation, runoff, infiltration, drainage, depth to water table and flow patterns in the water table.

Moreover, a hydrochemical evaluation may be conducted to determine surface aeration, pH and composition of pore waters and pore gases. The hydrogeological and

hydrochemical description provide information for computer modeling of flow and transport pathways at a petroleum-contaminated site, which may guide in the selection of an appropriate remediation strategy and the evaluation of the probability of a successful cleanup of the site using the strategy developed. Figure 1.1 depicts the potential release mechanisms from contaminated soils.

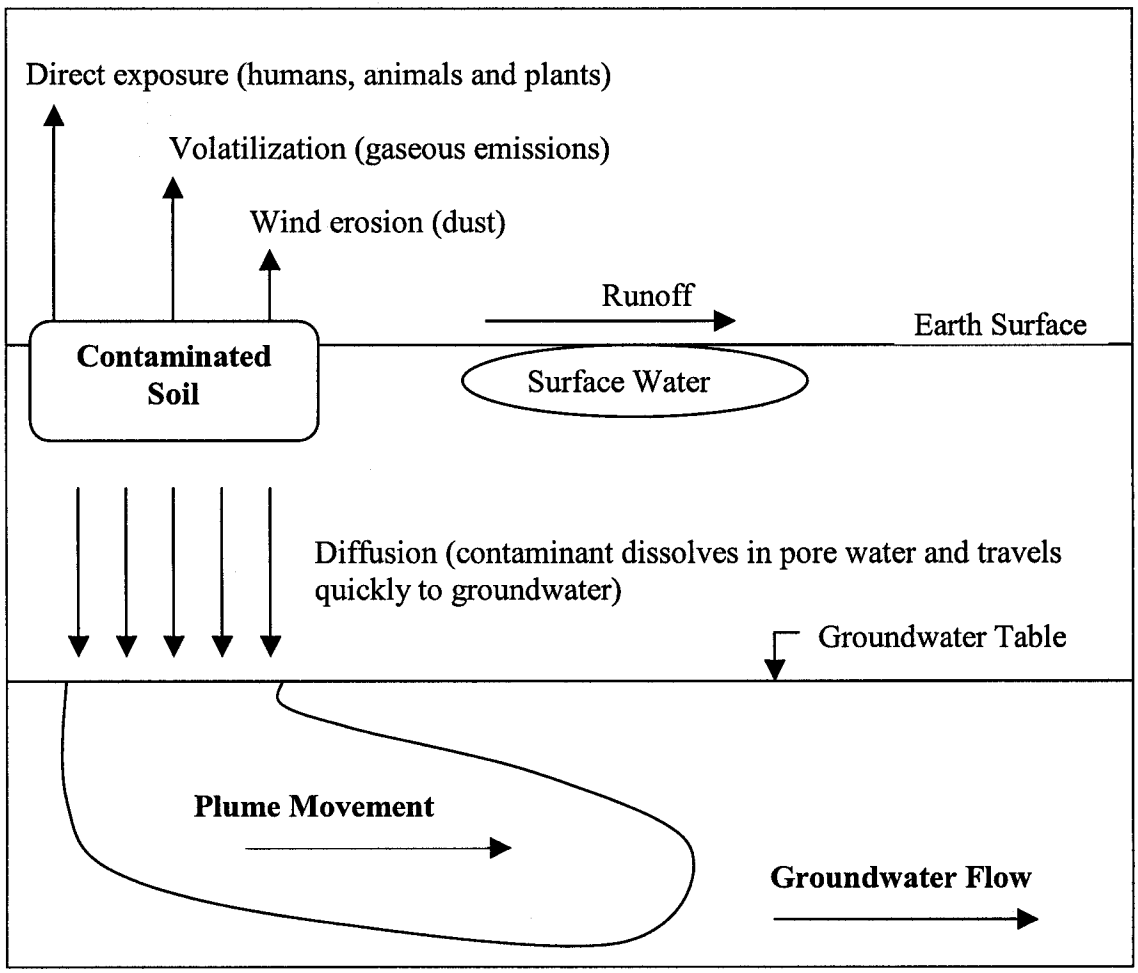


Figure 1.1 Potential release mechanisms of contaminated soils (Modified from Demque, 1994).

1.2 Assessment of Petroleum Contaminated Sites

Contaminated site assessment involves identifying the existence, source, nature and extent of contamination by toxic and hazardous substances and the determination of the threat posed to human health or the environment by the contamination.

Petroleum contaminated site assessment involves a series of assessments and investigations as briefly discussed below (Environment Canada, 2002); and as depicted in Figure 1.2.

Step #1 - Preliminary Site Assessment: This involves the collection of sampled field data to assist in the evaluation of a contaminated site through physical site characteristics, facility characteristics and contaminant characteristics. (Environment Canada, 2002). The geology, hydrology, soil characteristics and ecological processes of the site define the physical site characteristics. Facility characteristics involve the current and historical description of the site along with its facilities while the review and identification of the potential contaminants released to the environment is related to contaminant characterization.

Step # 2 - Field Investigation- Site Screening Methods: The purpose of field investigations is to define and delineate the contaminants present and the general extent and location of contamination using geophysical and soil vapour surveys methods. These field investigations include mapping of conductive leachates and location, depth, distribution and horizontal extent of contaminant plume; mapping of geohydrologic features (lateral and vertical changes); location of boundary definition of buried trenches location and definition of buried metallic objects (e.g. drums, utilities).

Step # 3 - Comprehensive Subsurface Investigation: This is related to the use of test pits and borehole and well construction to establish the site-specific data for analysis. This permits the visual characterization of soils, investigation of free product and residual soil contamination, permeability testing and the development of a three dimensional hydrogeological model.

Step # 4 - Analysis of Field Samples: The analysis of samples and data from the field and subsurface investigations provides information regarding the toxic levels and the environmental fate and transport mechanisms of contaminants within the environmental media. It also involves identifying the exposed population or potential targets and the potential exposure routes/pathways for contaminants.

Step # 5 - Remedial Investigation: The development of an environmental risk assessment is undertaken to ascertain if remedial action is necessary and what type of remedial action should be taken.

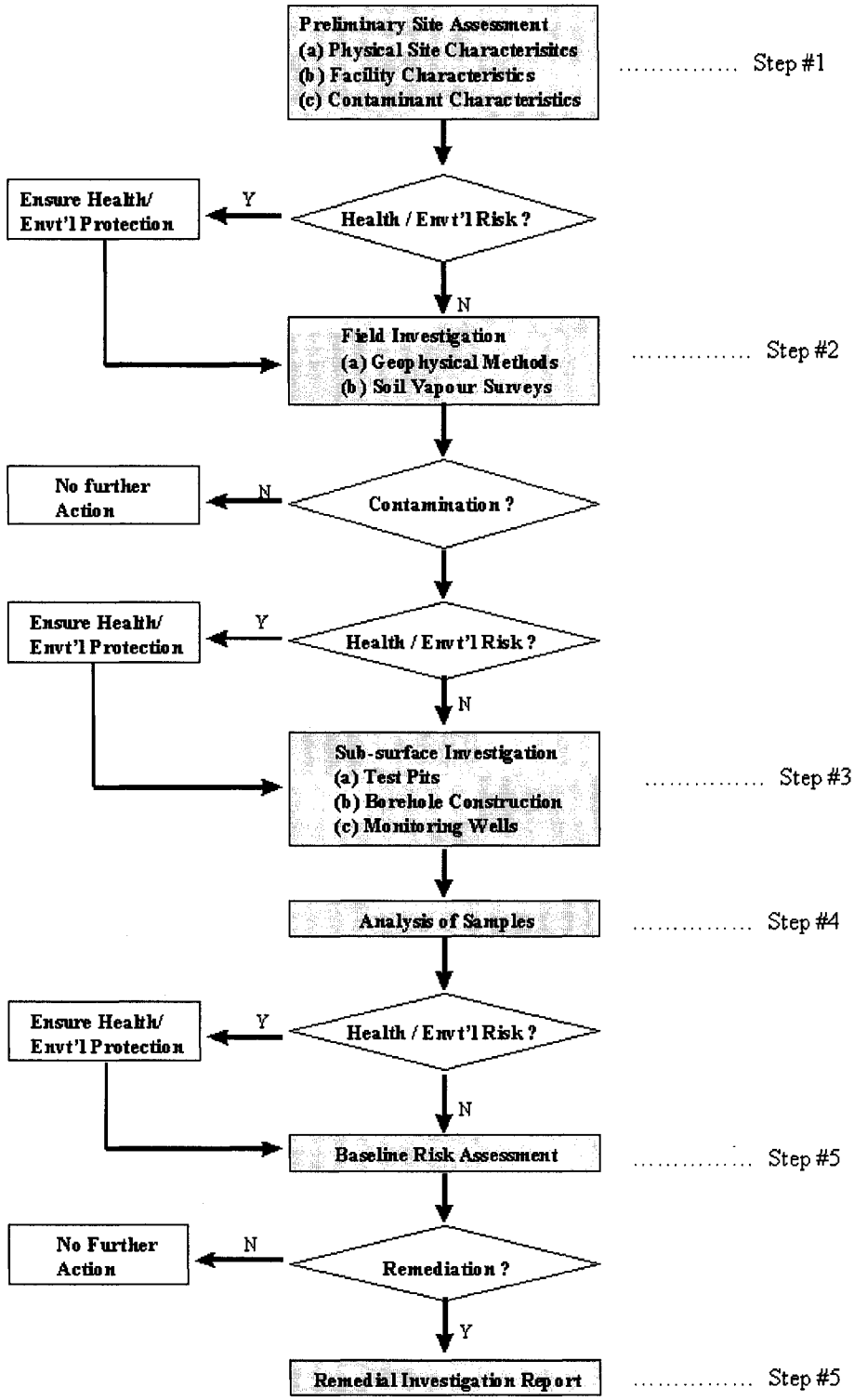


Figure 1.2: Flowchart outlining a site assessment procedure. (Environment Canada, 2002)

1.3 Remediation Techniques for Petroleum Contaminated Soil

Hydrocarbon pollution of the natural environment has been an important issue in recent years. Soil and groundwater contamination by petroleum products due to the thousands of leaking underground storage tanks is of major concern and the contamination of the environment by petroleum hydrocarbons is both widespread and frequent. (Cole, 1994).

Diesel fuel contaminated soil is a major environmental concern which is considered as the second most frequently treated contaminant after benzene at the United States Environmental Protection Agency superfund projects (Zytner, 2001) and a good typical source is leaking underground storage tanks at service stations (Atlas, 1995). This had led to a demand for further studies in the investigation, assessment, management and remediation of diesel-contaminated sites in the field of petroleum contamination.

A variety of options are available to remediate environmental impacts, depending on the characteristics and concentrations of the pollutants of concern and these remediation techniques include any of the following (USEPA 2004, Riser-Roberts, 1998).

Soil Vapour Extraction: Soil vapour extraction (SVE), also known as soil venting or vacuum extraction, is an situ remedial technology that reduces concentrations of volatile constituents in petroleum products adsorbed to soils in the unsaturated (vadose) zone.

Bioventing: Bioventing is an in-situ remediation technology that uses indigenous microorganism to biodegrade organic constituents adsorbed to soils in the unsaturated zone.

Biopiles: Biopiles, also known as biocells, bioheads, biomounds, and compost piles involves heaping contaminated soils into piles (or "cells") for biodegradation within the soils by microbial activities.

Landfarming: Landfarming, also known as land treatment or land application, is an above-ground remediation technology, which involves spreading excavated contaminated soils in a thin layer on the ground surface and stimulating aerobic microbial activity within the soils through aeration and/or the addition of minerals, nutrients and moisture.

Low-temperature Thermal Desorption (LTTD): This is known as low-temperature thermal volatilization, thermal stripping, or soil roasting. LTTD is an ex-situ remedial technology that uses heat to physically separate petroleum hydrocarbons from excavated soils.

Air Sparging (AS): This technology, which is also known as "in situ air stripping" and "in situ volatilization," involves the injection of contaminant-free air into the subsurface saturated zone, enabling a phase transfer of hydrocarbons from a dissolved state to a vapour phase.

Biosparging: In biosparging, air (or oxygen) and nutrients (if needed) are injected into the saturated zone to increase the biological activity of the indigenous microorganisms.

Monitored Natural Attenuation: This refers to the reliance on a carefully controlled and monitored site cleanup approach and natural attenuation processes to achieve site-specific remediation goals within a time frame.

In-Situ Groundwater Bioremediation: In-situ groundwater bioremediation is used to degrade organic constituents that are dissolved in groundwater and adsorbed onto the aquifer matrix.

Dual-Phase Extraction: Dual-phase extraction, also known as multi-phase extraction, vacuum-enhanced extraction, or bioslurping, is an in-situ technology that uses pumps to remove various combinations of contaminated groundwater, separate-phase petroleum product, and hydrocarbon vapour from the subsurface.

Enhanced Aerobic Bioremediation: Enhanced aerobic bioremediation technologies are used to accelerate naturally occurring in-situ bioremediation of petroleum hydrocarbons, and some fuel oxygenates such as methyl tertiary-butyl ether (MTBE) by indigenous microorganisms in the subsurface.

Chemical Oxidation: Petroleum contaminant decomposition and in-situ destruction may be accomplished using chemical oxidation technologies, in which a variety of chemical oxidants and application techniques can be used to bring oxidizing materials into contact with subsurface contaminants to remediate the contamination.

In this study, a combination of a controlled form of monitored natural attenuation and enhanced aerobic bioremediation was employed in reducing the concentrations of hydrocarbon in soils through the use of biodegradation.

1.4 Objectives of This Study

The objectives of this study were to;

1. Design and carry out an in-situ and aerobic form of bioremediation.

2. Investigate the biodegradation of diesel-contaminated soil under various treatments.

3. Compare the effects of nutrients, inocula and bulking agents additions on the removal rate of diesel-fuel in soils.

4. Study the interactions among the factors (nutrients, inocula and bulking agents) and the significance of each factor in the biodegradation experiments.

5. Evaluate which processes are responsible for the removal of diesel from contaminated soil.

6. Predict the best treatment combination or set of conditions for the bioremediation of diesel-fuel contaminated soil.

1.5 General Outline

The content of this thesis is organized into six chapters that are presented as follows:

- Chapter 1 includes an introduction to petroleum contaminated Sites, assessment of petroleum-contaminated sites, remediation techniques for petroleum-contaminated soil, objectives of this study and a general outline.
- Chapter 2 is a review of the origin of hydrocarbons in soils, fate and transport of hydrocarbons in soil, the composition of petroleum hydrocarbons, soil bioremediation and bioremediation of petroleum hydrocarbons
- Chapter 3 presents the materials and methods employed in the experiments.
- Chapter 4 is the bioremediation experimental design.
- Chapter 5 contains the results obtained in this research study and the discussion.
- Chapter 6 presents the conclusions and recommendations.

Chapter 2

Literature Review

2.1 Origin of Hydrocarbons in Soil

Petroleum hydrocarbons are the most common contaminants found in soils and groundwater and they occur as a result of either a deliberate or accidental release of petroleum into the environment. These discharges could come from any of the following:-

-Leaking underground storage tanks (USTs)

-Oil Production and Exploration

-Petroleum Refining

-Oil transportation and distribution

-Overfills and spills while filling tanks

-Aboveground tanks, terminals and pipelines.

-Pumps or dispensers

-Fuel lines between tanks and pumps

The anthropogenic activities of the present industrial society are a major source of hydrocarbons in the soil environment. There are however other natural sources of hydrocarbons (biogenic sources) and, these include seeps from oil deposits and the degradation of organic matter within the soil. It has been reported that certain organisms, e.g. higher plants are capable of synthesizing hydrocarbons (Langley et al., 2003) which eventually find their way into the soil environment.

Petroleum hydrocarbon products are water-immiscible, and are referred to as light non-aqueous phase liquids (LNAPL). Examples include gasoline, diesel, kerosene and fuel oil. The chemicals of concern found at petroleum-contaminated sites, depend upon the type of petroleum products (diesel, gasoline etc) originally released into the environment. Following release, petroleum hydrocarbons will exit in a combination of solid, liquid, dissolved, and vapour phases.

2.2 Fate and Transport of Hydrocarbons in Soil

A bioremediation protocol cannot be developed without a thorough site characterization, and an evaluation of the hydrogeological conditions, which entails a detailed description of the hydrology, geology and geochemistry of the unsaturated and saturated zones, which is useful for defining the pathways of contaminant transport, is necessary (Rosenbaum-Wilkinson, 1994). Hydrocarbons may be associated with organic matter and can undergo series of processes during transport within the soil as illustrated in Figure 2.1.

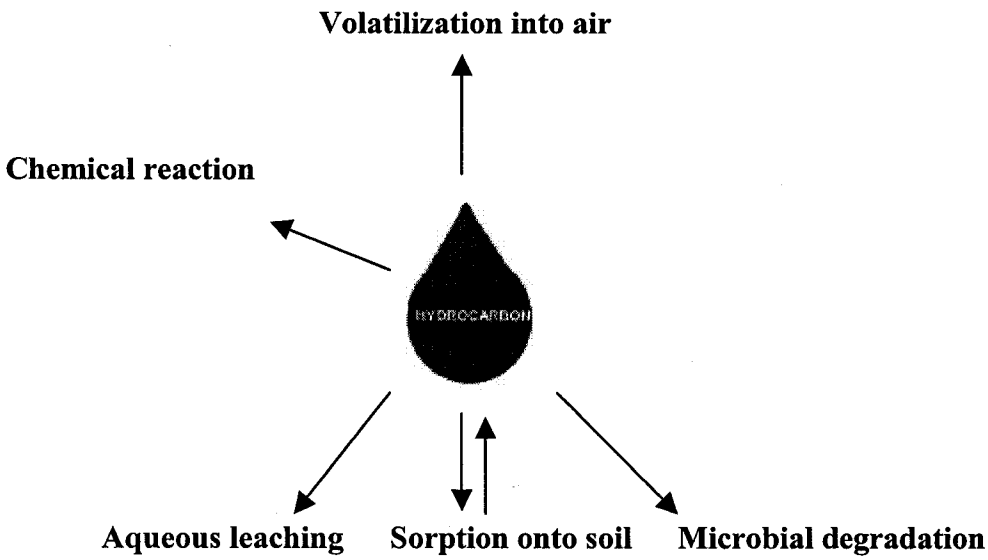


Figure 2.1: Processes undergone by hydrocarbons in soils. (Langley et al., 2003)

Volatilization: Volatilization into air involves the loss of volatile constituents of a petroleum product. Volatility is a function of the vapor pressure of a compound which defines the propensity of a chemical to partition to air and migrate as a vapor.

Chemical reaction: Hydrocarbon constituents undergo chemical reactions as well as photolytic or photo-oxidation reactions with the soil, that alter the hydrocarbon structure and limit its migration with the pours medium.

Aqueous leaching: As water, from rain, flooding or other sources, seeps into the ground, it can dissolve chemicals and carry them into the underground water supply and contributing to groundwater contamination.

Sorption: Sorption of hydrocarbon to mineral and organic matter contents in soils, limit the bioavailability of contaminants. Bioavailability depends upon the concentration of the

contaminant in the solution phase, and the solution phase is dependent upon the ease with which the contaminant moves from fixed states.

Microbial degradation: Organic compounds are readily degraded by indigenous microbes found in many natural settings (e.g., soils, groundwater, and ponds). Biodegradation occurs as microbes use organic compounds as a source of energy.

These processes result in an alteration of the hydrocarbon; however, hydrocarbons that are most strongly sorbed onto soil organic matter are most resistant to loss by the other processes. An alteration leads to "weathering" of the hydrocarbon mixture. Weathering refers to biological, chemical and physical processes that result in an accompanying change in the hydrocarbon composition and a preferential transport of certain fractions to other environmental compartments. (Loehr et al., 2001).

Compounds with low water solubilities and high octanol/water partition coefficients will be adsorbed more strongly to solids and are generally less biodegradable. The octanol/water partition (K_{ow}) coefficient is defined as the ratio of a compound's concentration in the octanol phase to its concentration in the aqueous phase of a two-phase system. Highly water soluble compounds tend to have low adsorption coefficients for soils and tend to be readily biodegradable (Crawford et al., 1996).

The bulk hydrocarbons in soil penetrate through the soil surface via the most permeable path. If the soil has a high clay content with a low permeability, the non-aqueous phase liquids (NAPLS) may pond on the soil surface. NAPLS are liquids that are sparingly soluble in water, and because they do not mix with water, they form a separate phase. However a sandy soil may allow rapid infiltration. The infiltration of NAPLS is a

function of their viscosity. The infiltration of oil is also dependent on the following primary properties; surface tension, mass and viscosity.

2.3 Composition of Petroleum Hydrocarbons

According to Nadim et al., (2000), "crude oil is a complex mixture of hydrocarbons". Its elemental composition is carbon-hydrogen, with variable quantities of oxygen and sulphur and trace amounts of nitrogen, metals and other elements. The average composition of crude oil is 83% carbon (C) and 12% hydrogen (H), and 5% sulfur (S), oxygen (O) and nitrogen (N).

Petroleum hydrocarbons (PHC) refer to the hydrogen and carbon-containing compounds that originate from crude oil. PHC can be divided into two main groups namely aliphatic hydrocarbons and aromatic hydrocarbons as illustrated in Figure 2.3. Aliphatic hydrocarbons are organic compounds that do not contain a benzene ring while aromatic hydrocarbons are compounds with benzene or similar structural features. Benzene is made up of a ring of six carbon atoms with variable single and double carbon-carbon bonds.

Aliphatic hydrocarbons can be divided into two categories; saturated hydrocarbons and unsaturated hydrocarbons. Saturated Hydrocarbons include the alkanes and the cycloalkanes i.e. hydrocarbons in which all carbon atoms are bonded to the maximum number of hydrogen atoms. Unsaturated hydrocarbons include the alkenes and the alkynes i.e. hydrocarbons that do not contain the maximum number of hydrogen atoms for a given carbon atom framework.

Alkanes, also called paraffins, are without any carbon-carbon double or triple bonds and have the general formula C_nH_{2n+1}. Cycloalkanes are branched chained saturated hydrocarbons with the general formula C_nH_{2n}. Alkenes are also known as olefins and contain a carbon-carbon double bond, while the alkynes contain a carbon-carbon triple bond. Aromatic hydrocarbons are unsaturated hydrocarbons that contain benzene rings, and can be monocyclic or polycyclic (fused hydrocarbons).

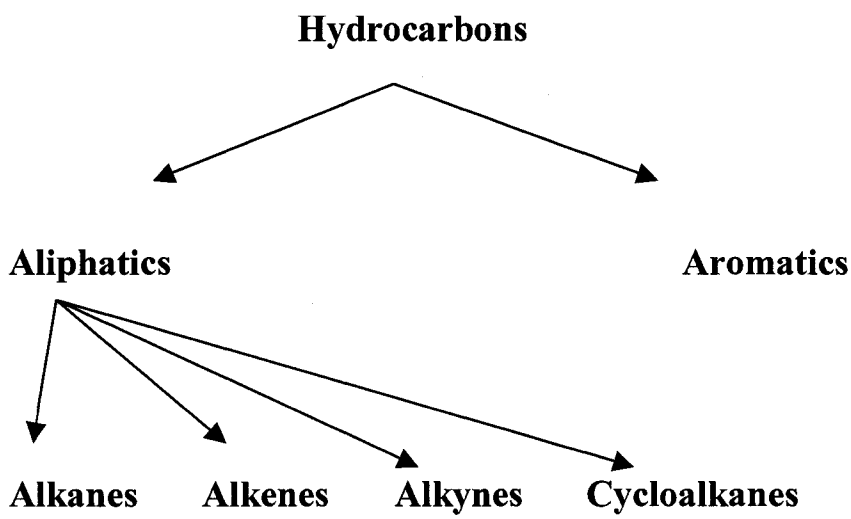


Figure 2.2: Classification of Hydrocarbons

Petroleum products are complex mixtures of hundreds of hydrocarbon compounds, ranging from light, volatile, short-chained organic compounds to heavy, long chained, branched compounds. The exact composition of petroleum products varies depending upon (1) the source of the crude oil (crude oil is derived from underground reservoirs, which vary greatly in their chemical composition) and (2) the refining practices used to produce the product.

A fraction of petroleum e.g. gasoline has a boiling point of −12 °C to 200°C, and contains over 1,200 different hydrocarbons, with carbon numbers ranging from C_3 to C_{12}. Other fractions of petroleum include diesel and jet fuel and these have a boiling point ranging from 170°C to 340°C and contain carbon numbers ranging from C_9 to C_{28}. Heavy petroleum products such as lubricating oil, paraffin wax and asphalt, have boiling points of over 350°C. This is illustrated in Figure 2.4.

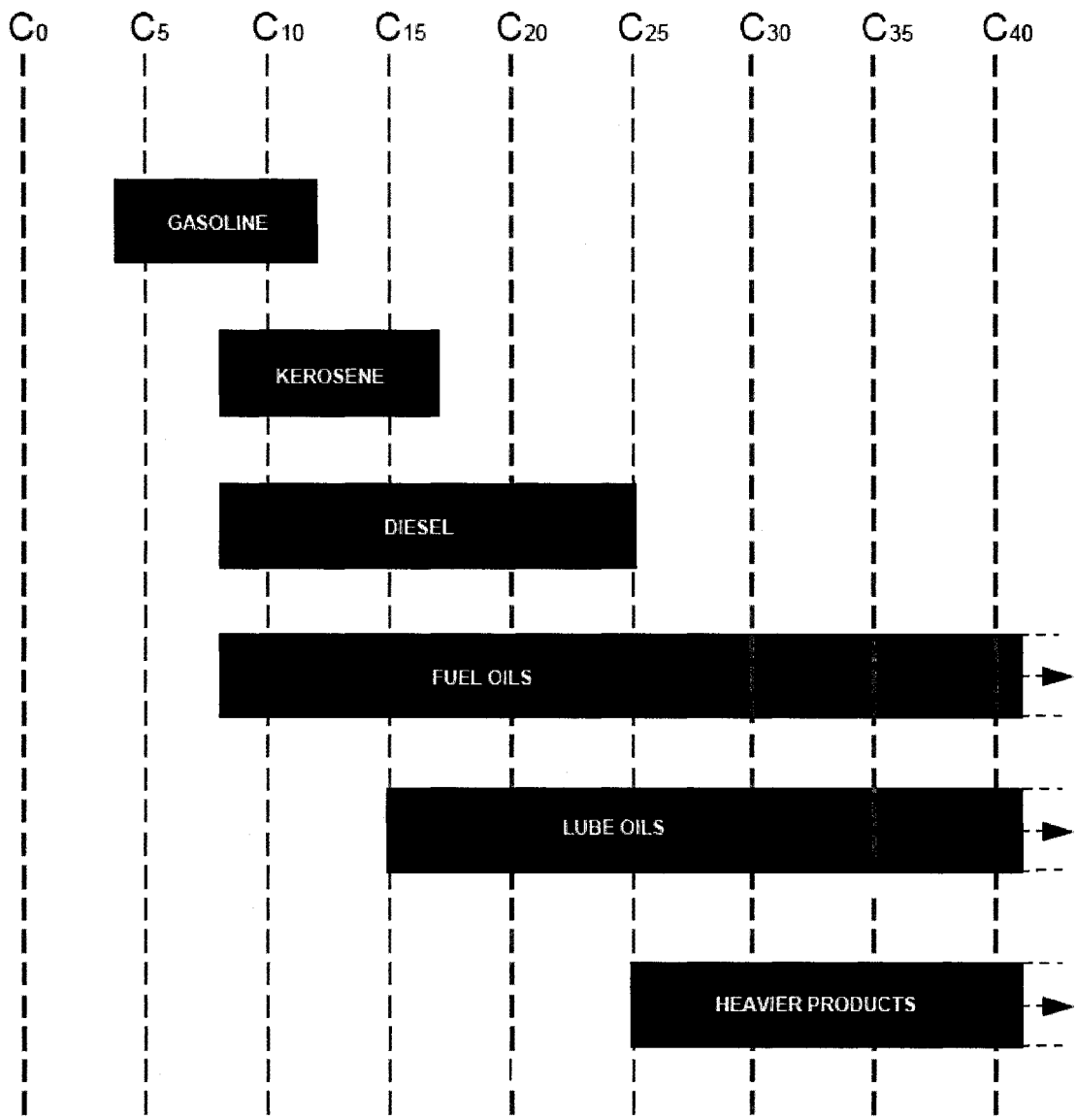


Figure 2.3: Carbon Number Ranges for Petroleum Products (Dames & Moore 1997).

A variety of hydrocarbon components are found at contaminated sites. Table 2.1 lists the properties of a range of simple paraffin alkanes that could be found at a

contaminated site. Table 2.2 also gives some of the physical properties of aromatic molecules that might occur at contaminated sites.

Table 2.1 Simple Paraffin Alkanes

Molecular Formula	**Name**	**Boiling Point (°C)**	**Melting Point (°C)**	**Density at 20°C**
C_6H_{14}	*n*-Hexane	69	-94	0.658
C_8H_{18}	*n*-Octane	126	-95	0.702
$C_{10}H_{22}$	*n*-Decane	174	-32	0.747
$C_{12}H_{26}$	*n*-Dodecane	215	-12	0.768
$C_{16}H_{34}$	*n*-Hexadecane	287.5	18	0.755(at mp)
$C_{20}H_{42}$	*n*-Eicosane	205	36.7	0.778(at mp)
$C_{30}H_{62}$	*n*-Triacontane	449.7	66	0.775
$C_{35}H_{72}$	*n*-Pentatriacontane	490	74.6	0.781

Table 2.2 Some Aromatic Compounds

Molecular Formula	**Name**	**Boiling Point (°C)**	**Melting Point (°C)**
C_6H_6	Benzene	18	5.5
$C_{10}H_8$	Naphthalene	218	80.3
$C_{14}H_{10}$	Phenanthrene	338	100.5
$C_{18}H_{12}$	Chrysene	448	253
$C_{20}H_{12}$	Benzo(a)pyrene	310-312	179
$C_{22}H_{12}$	Benzo(g,h,i)perylene	542	278

The physical parameters for the total petroleum hydrocarbons (TPH) aliphatic fractions based on correlations to the boiling points indices for aliphatic hydrocarbons are presented in Table 2.3.

Table 2.3 Physical Parameters for TPH Aliphatic Fractions

Carbon Equivalent Fraction	Log S_w (mg L^{-1})	Vapour Pressure (atm) at 26-30°C	Henry's Law Constant (dimensionless)	Log K_{oc}
C_5-C_6	1.56	3.5×10^{-1}	47	2.9
$C_{>6}$-C_8	0.73	6.3×10^{-2}	50	3.6
$C_{>8}$-C_{10}	-0.36	6.3×10^{-3}	55	4.5
$C_{>10}$-C_{12}	-1.46	6.3×10^{-4}	60	5.4
$C_{>12}$-C_{16}	-3.12	7.6×10^{-5}	69	6.7
$C_{>16}$-C_{35}	-5.60	1.1×10^{-6}	85	8.8

Henry's Law Constant (dimensionless): The Henry's Law constant is a proportionality constant that relates the concentration of a volatile chemical in air to its concentration in an aqueous solution at equilibrium.

Vapor pressure: The pressure exerted by a vapor in equilibrium with the liquid or solid phase of the same substance at a given temperature.

Carbon Matter Partition Coefficient, K_{oc}: (ml/g): The carbon matter partition coefficient describes partitioning of a chemical between the aqueous phase and soil in contact with water. The carbon matter partition coefficient is used to estimate the absorption coefficient, K_d.

Water Solubility (S_w ; mg/L): The solubility of a pollutant in water is expressed in units of mg/L at a temperature in range of 20 to 30 °C.

The physical parameters for the TPH aromatic fractions based on correlations to the boiling points indices for aromatic hydrocarbons are presented in Table 2.4.

Table 2.4 Physical Parameters for TPH Aromatic Fractions

Carbon Equivalent Fraction	Log S_w (mg L^{-1})	Vapour Pressure (atm) at 26-30°C	Henry's Law Constant (dimensionless)	Log K_{oc}
C_5-C_7	2.34	1.1×10^{-1}	1.5	3.0
$C_{>7}$-C_8	2.11	3.5×10^{-2}	8.6×10^{-1}	3.1
$C_{>8}$-C_{10}	1.84	6.3×10^{-3}	3.9×10^{-1}	3.2
$C_{>10}$-C_{12}	1.40	6.3×10^{-4}	1.3×10^{-1}	3.4
$C_{>12}$-C_{16}	0.76	4.8×10^{-5}	2.8×10^{-2}	3.7
$C_{>16}$-C_{21}	-0.19	1.1×10^{-6}	2.5×10^{-3}	4.2
$C_{>21}$-C_{35}	-2.18	4.4×10^{-10}	1.7×10^{-5}	5.1

2.4 Chemistry of Petroleum Hydrocarbons (Diesel Fuel)

Diesel fuels, which are sometimes called fuel oils, are regarded as middle distillates of crude oil consisting of hydrocarbons with carbon numbers within the range of C_9 to C_{20} (Demque et al., 1997). Diesel fuel is a complex mixture of hydrocarbons consisting of approximately 30% alkanes, 45% cyclic alkanes, 24% aromatics (Frankenberger et al., 1989) and 4% polyaromatic compounds (Heath et al., 1993).

There are basically two types of diesel fuels; these are diesel fuel # 1 and diesel fuel # 2. Diesel fuel # 1 is kerosene, which does not contain benzene and polycyclic aromatic hydrocarbons {PAHs}. Diesel fuel # 2 has the carbon number ranges from C_{11} to C_{22} and a lower percentage composition of straight chain-chain fractions. The typical chemical and physical properties of diesel fuel are presented in Table 2.5.

Table 2.5 Chemical and physical properties of diesel fuel (Custance et al., (1992)

Diesel Fuel Properties	**Value**
Density (g/cm^3)	0.84
Aqueous solubility (mg/l)	0.20
Vapour pressure (atm)	3.95×10^{-5}
Diffusion coefficient in air {cm^2/s)	4.63×10^{-2}
Henry's law constant (atm-m^3/mol)	4.2×10^{-2}
Carbon matter partition coefficient	$10^{3.04}$

2.5 Soil Bioremediation

Bioremediation of contaminated soil and sediments involves the use of microorganisms to convert organic contaminants to carbon dioxide and water (a process known as mineralization) or to a less harmful compound. Bioremediation has been proven to be a cost effective and successful method for the remediation of sites

contaminated with a wide variety of organic and inorganic compounds (Alexander 1999; Crawford et al., 1996; Suthersan, 1997).

Various forms of treatment technologies have been employed in the bioremediation of diesel-contaminated soil and are reported in the literature. Frankenberger et al., (1989) reduced the petroleum constituents' concentration at a diesel-contaminated site from 1500 mg/kg of soil to less than 1 mg/kg by injected nutrients (nitrogen and phosphorus) and hydrogen peroxide was added to provide molecular oxygen to the subsurface microflora in degrading the petroleum. More so, Cunningham et al., (2000) investigated several factors (bioaugmentation; biostimulation via inorganic fertilizer and bulking agents) that influenced the removal rate of a diesel-contaminated site due to leakage from stabled Diesel Motor Unit (DMU) sets in an ex-situ treatment of a contaminated soil using windrow and biopiles.

Zytner et al., (2001) used field and laboratory studies to study the influence of temperature on two diesel contaminated soils using bioreactors under controlled conditions where degradation was attributed to be mostly the result of biodegradation, with minimal volatilization and negligible leaching. The intrinsic biodegradability of fuels such as gasoline or diesel oil was determined by Marchal (2003) using a reference aerobic microflora from an urban waste treatment plant, where gasoline exhibited a high intrinsic biodegradability (96%) and the commercial diesel oil was between 60 and 73%. Demque et al., (1997) conducted a field study to examine biodegradation rates of diesel fuel in Petawawa sand, under different treatment conditions (bisotimulation, tillage rates and introduction of acclimatized microorganisms) over a 14 week testing period. It was

concluded that biostimuation by adding commercial fertilizer was the most important factor in this land treatment test program where a reduction of 61-83% and 50% for half-life and final TPH concentrations were obtained respectively, compared with tests without biostimulation.

The accumulation and persistence of toxic materials in soil is a major problem today. Bioremediation of soil contaminated with organic chemicals is a viable method for clean-up of hazardous sites (Kosaric, 2001). Quite a number of soils act as natural adsorption systems for petroleum hydrocarbons and for this reason, soils with silt and clay are more difficult to remediate than soils with only sands. (Russell, 1992).

2.5.1 Types of Soil Bioremediation Strategies

Many different soil bioremediation methods have been applied or proposed, but they all involve the stimulation of microbial growth to degrade the contaminants of concern. Soil bioremediation may be broadly divided into in situ and ex situ bioremediation strategies as well as aerobic and anaerobic bioremediation (Cunningham et al., 2000 and Vidali, 2001). Aerobic bioremediation refers to bioremediation in the presence of oxygen while anaerobic bioremediation is bioremediation in the absence of oxygen. Previous studies have demonstrated the effective use of bioremediation treatment techniques for petroleum-contaminated soils (Rahman et al., 2002; Jorgensen et al., 2000; Kirchmann 1998).

In situ bioremediation refers to treatments not requiring the excavation of contaminated soil prior to treatment. In situ bioremediation is categorized as either engineered bioremediation or intrinsic bioremediation. Intrinsic bioremediation is also termed natural attenuation according to the US EPA (1999), because it involves the "use of natural processes to contain the spread of contamination from chemical spills and reduce the concentration and amount of pollutants at contaminated sites". Engineered bioremediation involves the introduction of microorganisms and nutrients, termed bioaugmentation and biostimulation, respectively.

Ex situ treatments include land farming, biopiles/composting and bioreactors. Bioreactors represent the controlled form of bioremediation, as soil is slurried with water and is treated in a specifically designed reactor, where the conditions for biodegradation are optimized. A schematic diagram of what bioremediation entails is depicted in Figure 2.5.

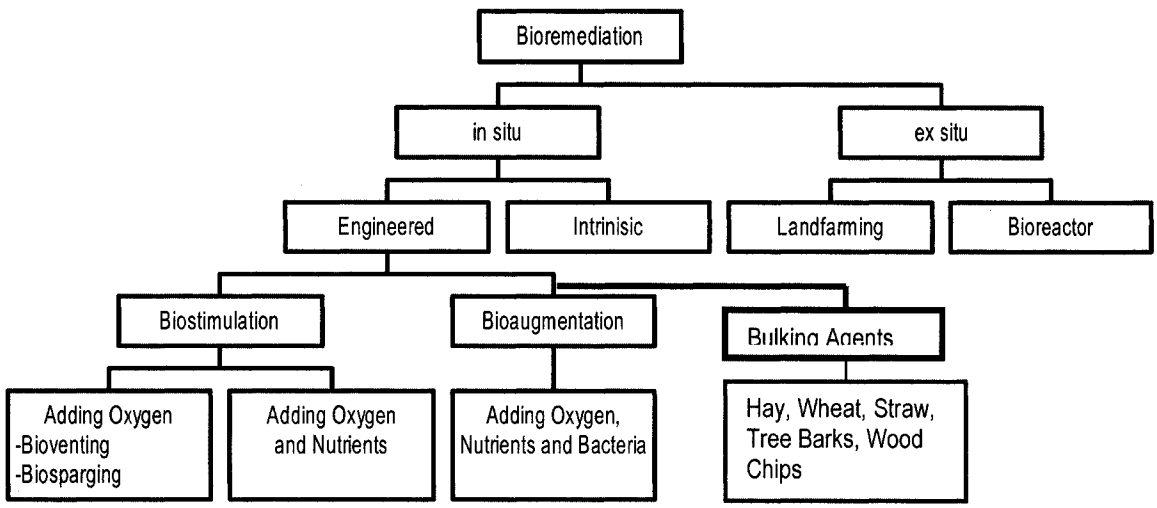


Figure 2.4 Diagram of Bioremediation

Introducing conditions more favorable to the activities of the microorganisms enhances the rate of bioremediation. In most cases, the remediation of hydrocarbon contaminated soils using bioremediation techniques involves biostimulation or nutrient addition to stimulate the indigenous microbial population (Cunningham et al., 2000), bioaugumentation or introduction of exogenous degrading microbial strains (Marquez-Rocha et al., 2001) or amending the soil with bulking agents such as wood chips, sawdust, leaves, hay, wheat bran, or shredded rubber tires to increase soil porosity. (Cookston, 1995; Vasudevan et al., 2001).

Bulking agents are usually materials of low density that when added to soils, lower the soil's bulk density, increase porosity, may increase oxygen diffusion and may help form water stable aggregates (Rhykerd et al., 2001). Such changes lead to an increase in soil aeration and microbial activity (Hillel, 1980). Morgan et al. (1993) showed that bulking of contaminated soil with chopped wheat, straw, hay, wood chips, pine bark, peat and loam enhanced remediation of 3, 4- dichloroanaline and benzo(a)pyrene. Another laboratory study involving bermudagrass and alfalfa were found to enhance biodegradation of crude oil in soil (Chang et al., 1998). Tillage and bulking with wheat bran were also found to influence the disappearance of hydrocarbons (Rhykerd et al., 2001).

Biostimulation with inorganic salts, commercial fertilizers, horse manure, poultry litter or domestic sewage sludge have been found to double the removal rate of hydrocarbons in soil (Demque et al., 1997; Williams et al., 1999; Cunningham et al., 2000; Gallego et al., 2001).

There have been confusing reports on the efficacy of bioaugmentation for remediation of contaminated soils. Demque et al. (1997) reported that it was unnecessary and undesirable to apply acclimated indigenous microorganisms to the soil. Moller et al. (1995) reported negative effects on diesel-contaminated soil bioremediation using commercial bioaugmentation products. It has been generally stated that bioaugmentation is best suited for certain special cases where intrinsic bioremediation or biostimulation does not work because of insufficient or non-acclimatized bacterial population (Cunningham et al., 2000) and where the use of inocula may be beneficial to recalcitrant compounds (Jorgensen, 2000).

2.5.2 Soil Processes Affecting Bioremediation

In soil bioremediation, the rate-limiting step is often the desorption of contaminants, since sorption to soil particles and organic matter in soils can determine the bioavailability of organic pollutants. (Crawford et al., 1996). Bioavailability is the degree to which a pollutant is available for biologically mediated transformations.

2.5.2.1 Transport

The transport of pollutants within the soil environment can be by leaching toward the groundwater, runoff toward surface water or by volatilization into the air. (Adriano et al., 2000). The transport process often involves advection, dispersion and diffusion. Advection is controlled by the flux of water through the soil while dispersion is caused

by the heterogeneity in the pore size distribution of the porous media. Diffusion is a function of the concentration gradient.

Volatilization describes the escape of a chemical from the soil environment into the atmosphere. The chemical moves either in the liquid or vapour phase to the soil surface and then escapes into the atmosphere. The volatility of a chemical does not influence its potential for biodegradation (Norris 1994). Highly volatile pollutants may possess low aqueous solubility and this may reduce the effectiveness of bioremediation techniques.

2.5.2.2 Retention

Retention, otherwise known as sorption often reduces the bioavailability and degradability of the organic pollutants (Alexander 1999). Active soil particle surfaces for retention are those of clay materials, soil organic matter and oxides. The mechanisms of interaction between organic contaminants and clay particles include London-van der Waals forces, hydrophobic reactions, hydrogen bonding and charge transfer, ligand and ion exchanges and chemisorptions (Yong et al., 2004).

2.5.2.3 Transformation

The ultimate goal of a bioremediation design is to detoxify organic pollutants by biological means. This transformation process is a function of the concentration of the parent pollutant that is reduced to less toxic metabolites. A complete transformation entails the production of CO_2, NH_4, SO_2 and water. Microorganisms predominantly carry

out soil transformation process, however phytoremediation or use of plants, plays a significant role. The characterization of the pathways during pollutant degradation identifies the intermediary metabolites formed while the characterization of the kinetics of the pollutant degradation determines the degradation rates under a variety of environmental conditions.

2.5.3 Soil Factors Affecting Bioremediation

Since soil is a key component of a bioremediation system, the influence of the physical and chemical properties of soil is taken into account during a bioremediation design. A successful bioremediation strategy must take into consideration variations in soil properties across a landscape that is affected by soil contamination.

2.5.3.1 Soil Physical Environment

The soil physical environment defines the settings in which chemical and biological activities take place. A thorough characterization of the physical and geochemical properties of soils is highly essential in the design of a bioremediation plan for petroleum-contaminated soil. The fate of petroleum hydrocarbons in soil is largely dependent on the geochemical and geotechnical properties of soil. Geochemistry of the soil controls the nutrient availability while the geotechnical parameters control migration or retention of petroleum hydrocarbons.

Soil water, an important factor for bioremediation is controlled by the structure, porosity and texture of the soils at the site.(Cheng et al., 1999). Aerobic bioremediation in

soil requires adequate amounts of oxygen and water. Optimum moisture for effective aerobic bioremediation is between 30% and 80% of the water content available for plant usage (Baker et al., 1994).

Soil hydraulic conductivity and permeability affect the feasibility of bioremediation (Thomas et al., 1993). Hydraulic conductivities larger than 10^{-4} cm/s are adequate for transport of nutrients and pollutants. Poor results have been obtained when fractured rocks with low permeability are flushed with surfactants and cosolvents, due to the inability to deliver the flushing solutions to the contaminants (Strbak, 2000). Finely texture soils and sediments that possess low permeability will limit supply of nutrients and oxygen to microorganisms. A higher proportion of clay minerals with their high surface area and chemical reactivity may experience biofouling as a result of soil pores becoming plugged with microbial cells. (Thomas et al.,, 1993).

Soil temperature and soil moisture affect the kinetics of soil reactions, since microbial activities in soil involve enzymatic and biochemical processes that are temperature sensitive. Metabolic processes during biochemical activities double with each 10°C increase in temperature (Baker, 1994). Soil organic matter is an important source of nutrients for microorganisms; hence decreases in organic matter content with depth are often linked with decreases in microbial population density and decreased ability to degrade toxic chemicals (Mallawatantri et al., 1996). Moreover, a decrease in organic matter with depth can also reduce the soil's sorption capacity, hence increasing the mobility of the pollutant.

2.5.3.2 Soil Chemical Environment

The soil nature and properties and the concentration and extent of contamination are essential factors that must be assessed to determine the likelihood of successful bioremediation (Troy, 1994). Contaminated sites with a high contaminant concentration or high resistance to biological transformation are not ideal for soil bioremediation.

Soil chemical properties that influence contaminant fate, transport and biodegradation are those that affect the solubility and retention of the contaminants and thus govern soil biological activities. These include soil pH, cation and anion exchange capacities, mineral and organic matter contents, and the presence of nutrients, salts, minerals and heavy metals. Soil is composed of minerals and organic matter in the solid phase. The most chemically reactive soil mineral fraction is the clay-sized particles as they possess the largest surface area for interactions and they influence the sorptivity of a polar or less polar contaminant, while sand and silt particles have smaller total surface area and thus, they are less reactive chemically. Clay minerals are electrically charged and possess cation- and anion-exchange capacities that also influence the retention of polar chemicals and affect pH, thereby influencing metal speciation and nutrient availability for microorganisms.

Soil is rich in nutrients for microorganisms; these nutrients include macronutrients such as carbon (C), nitrogen (N), phosphorus (P), potassium (K), sulphur (S) as well as all micronutrients such as iron (Fe), copper (Cu), manganese (Mn) and boron (B). It is commonly assumed that the ratio between biological oxygen demand (BOD), ammonia nitrogen (NH_3-N) and ortho-phosphorus should be about 100:10:1 (Riser-Roberts, 1998).

Microorganisms require trace amounts of micronutrients and they are usually present in adequate concentrations in most soils for adequate microbial nutrition and so need no further attention in the design of a bioremediation process (Cookson, 1995). However, the effect of trace elements in bioremediation can be seen in their ability to affect soil geochemistry. Soil solutions with high concentration of Ca, Mg or Fe can promote precipitation of orthophosphate injected for nutritional supplement, which can clog well screens and water lines at a remediation sites (King et al., 1992). Moreover, H_2O_2 injected into soil to provide oxygen for microbial respiration can be decomposed instantaneously by the catalytic power of Fe, Cu and Mn at concentrations as low as 10mg/kg (King et al., 1992).

2.5.3.3 Soil Biological Environment

The natural biodegradability of polluted soil is a function of the soil microbial properties. Microbial characterization of soil includes enumeration of total heterotrophic microorganisms and contaminant specific oil degraders. Soils usually contain large numbers of native or indigenous microorganisms that are known to degrade petroleum hydrocarbons. (Gogoi et al., 2003). Hydrocarbon levels higher that 10% have been reported to have inhibitory effects on the soil microbial activities. (Husesemann, 1994).

Most biological activities occur in the surface soil horizon. Both soil physical and chemical environments affect soil biological activity. Soil conditions most conductive for aerobic microorganisms include a well-aerated environment, an abundant supply of nutrients and energy sources, a sufficient moisture supply and a favourable temperature

regime. When soil is saturated with water, oxygen diffusion into the soil matrix is limited, and the soil environment can become anaerobic, restricting activities of aerobic microorganisms. Anaerobic microorganisms thrive using organic substrates for energy in the presence of appropriate electron acceptors, which include NO^{3-}, SO_4^{2-}, reducible metal oxides, oxidized C, and S^{2-}. A complete degradation of a chemical in soil to its inorganic end products such as CO_2 and water usually involves the combined efforts of a mixed population of microorganisms including fungi, bacteria, algae and actinomycetes. (Cheng et al., 1999).

2.6 Bioremediation of Petroleum Hydrocarbons

Bioremediation technology is being utilized for the degradation of gasoline, diesel, jet fuel and heating oils in the soil matrix using the enzymes contained in microbial cells along with favorable soil and environmental conditions such as nutrients, oxygen, moisture and temperature (Dragun, 1998). The biodegradation of an organic chemical is the modification or decomposition of the chemical by soil microorganisms to produce ultimately microbial cells, carbon-dioxide (CO_2) and water (H_2O); this modification is carried out entirely by enzymes located within the microbial cells. Biodegradation is a biologically catalyzed reduction in complexity of chemicals, which leads to the conversion of much of the C, N, P, S and other elements in the original compound to inorganic products, a process also known as mineralization (Alexander, 1999). Figure 2.5 is a schematic diagram of biodegradation.

The transformation of a chemical after its collision with enzymes of the cells depends upon (1) the chemical binding to the enzyme and (2) conformational changes at the enzyme's active site (Dragun 1998). Bioremediation is the optimization of biodegradation. Bioremediation is an engineered process where the natural biodegradation of petroleum hydrocarbons by indigenous soil bacterial, fungi and protozoa is accelerated.

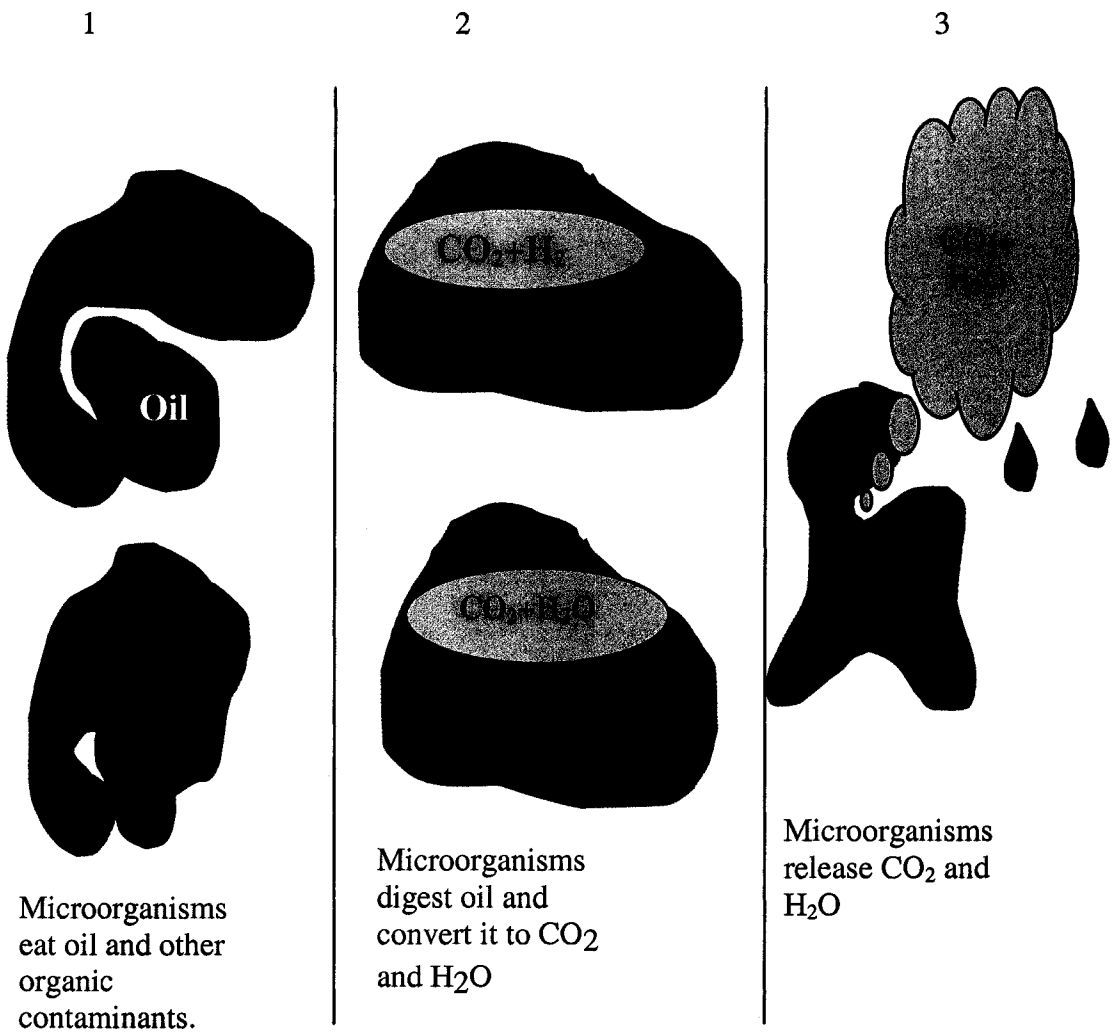


Figure 2.5: Schematic Diagram of Biodegradation (McCrary, 1998).

2.6.1 Requirements for Bioremediation

The general optmimum requirements for the degradation of contaminants are presented in Figure 2.6 in descending order of importance. Microorganisms are of paramount importance because they are capable of producing enzymes that will degrade the hazardous chemical (target compound). To achieve a sucessful biormediation protocol, the engineer must establish the limiting environmental conditions and then control these conditions for optimized bioremediation. Optimum environmental conditions for the degradation of contaminants are reported in Table 2.6.

Table 2.6 Environmental conditions affecting degradation (Vidali, 2001).

Parameters	**Condition required for microbial activity**	**Optimum value for an oil degradation**
Soil Moisture	25–28% of water holding capacity	30–90%
Soil pH	5.5–8.8	6.5–8.0
Oxygen Content	Aerobic, minimum air-filled pore space of 10%	10–40%
Nutrient Content	N and P for microbial growth	C:N:P = 100:10:1
Temperature (^{o}C)	15–45	20–30
Contaminants	Not too toxic	Hydrocarbon 5–10% of dry weight of soil
Heavy Metals	Total content <2000 ppm	<700 ppm
Type of Soil	Low clay or silt content	

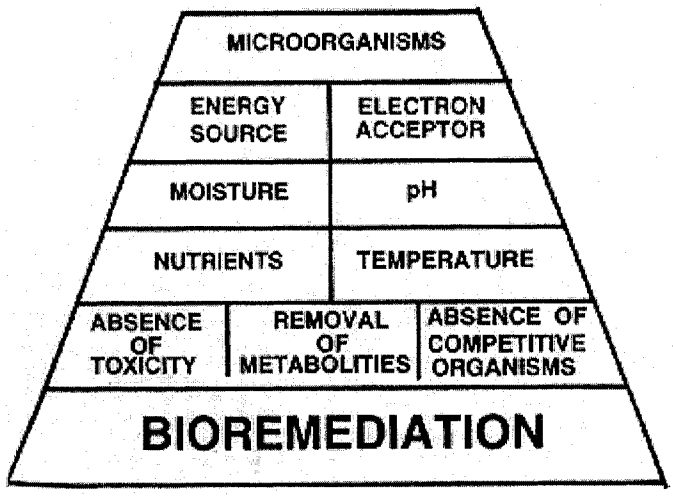


Figure 2.6: Requirements for Bioremediation (Cookson, 1995)

2.6.2 Factors Influencing the Success of Bioremediation

Physical, chemical and biological factors affect the efficacy of bioremediation for alleviation of contaminated sites (Edgehill, 1992). These factors include the following;

i. binding properties of the pollutants

ii. degree of mixing of inoculated cells

iii. oxygen availability

iv. presence of nutrients

v. microbial metabolism and growth kinetics

vi. solubility/availability of pollutant

vii. temperature

viii. presence of predator microorganisms that compete for nutrients within the soil matrix.

Chapter 3

Materials, Methods, Characterization and Interpretation.

3.1 Materials

3.1.1 Soils

Two different soils were obtained for the biodegradation experiments; a non-contaminated native soil from Memorial University of Newfoundland botanical gardens and a diesel-contaminated soil near the Memorial University of Newfoundland printing plant diesel generator storage tank. The soils were stored at 4°C in a refrigerator.

The soils were air dried and sieved using a #10 US (2.0 mm) sieve to remove gravel, stones, debris and chunks. The weight of the diesel-contaminated soil was 0.14 kg and the weight of the non-contaminated soil was 6.5 kg. Only a small fraction of diesel contaminated soil could be obtained (0.14 kg) during the time of sampling; the winter season. The two soils were mixed together to obtain a homogeneous mixture of 6.6 kg of mixed soil which was stored in a fish tank for four weeks and the small fraction of diesel

contaminated soil was needed and was used so that the diesel degraders in the soil can proliferate in the new soil mix. Deionised water was regularly added to keep the soil moist and provide the moisture content needed for the microorganisms to flourish in the new soil mix before the biodegradation experiments.

3.1.2 Hydrocarbons

The hydrocarbon source used for the biodegradation experiments was commercial diesel fuel purchased locally from an Ultramar filling station in St. John's, Newfoundland. The chemical and physical properties of commercial diesel fuel are given in Table 2.5.

3.1.3 Nutrients

Nutrients used for the biodegradation experiments were poultry manure obtained from Rushmore Farms, Whitbourne, Newfoundland and liquid cow manure obtained from Oceanview Farm, Bay Bulls, Newfoundland. Prior to the addition of the manures to the soil, a nutrient analysis of the manures was performed to estimate the organic carbon, nitrogen and phosphorus contents of the manures. Table 3.1 summarizes the results obtained.

Table 3.1 Organic Carbon, Total Nitrogen and Total Phosphorus Contents of the Manures

	Value	**Poultry Manure**	**Cow Manure**
Organic Carbon	(%)	14	2.8
Nitrogen	(%)	2.7	0.4
Phosphorus	(%)	0.2	0.14

3.1.4 Microorganisms

Two sources of microorganisms were used for the biodegradation experiments; the soil indigenous microorganisms that were originally present in the soil for the biodegradation experiments and a commercial microbial sample supplied by Universal Environmental Services, St. John's, Newfoundland. The microbial analysis of soil and commercial microbial samples was conducted and the procedure and results are clearly stated in Section 3.3.2.

3.1.5 Bulking Agents

The bulking agents used were Ottawa sand purchased from Fisher Scientific Incorporation and hay obtained from Runshmere Farms, Whitbourne, Newfoundland. The properties of the Ottawa sand are shown in Table 3.2. Hay is a form of carbohydrate (cellulose, a polymer of glucose).

Table 3.2: Properties of Ottawa sand

Properties	
Molecular Formula	SiO_2
Formula Weight	60.9
Melting Point (°C)	1610°C
Boiling Point (°C)	2230°C
Vapor Pressure (mm Hg)	N/A
Vapor Density (Air=1)	N/A
Solubility in Water	Insoluble
Appearance & Odor	White, yellow or tan crystals or granules; no odor
Specific Gravity (H2O = 1)	2.65
Percent Volatile by Volume (%)	N/A
Evaporation Rate (Butyl acetate =1)	N/A
Mesh size	590 microns

3.2 Experimental Procedure

A flow chart of the experimental procedure employed in this study is depicted in Figure 3.1. All experiments were done in duplicate.

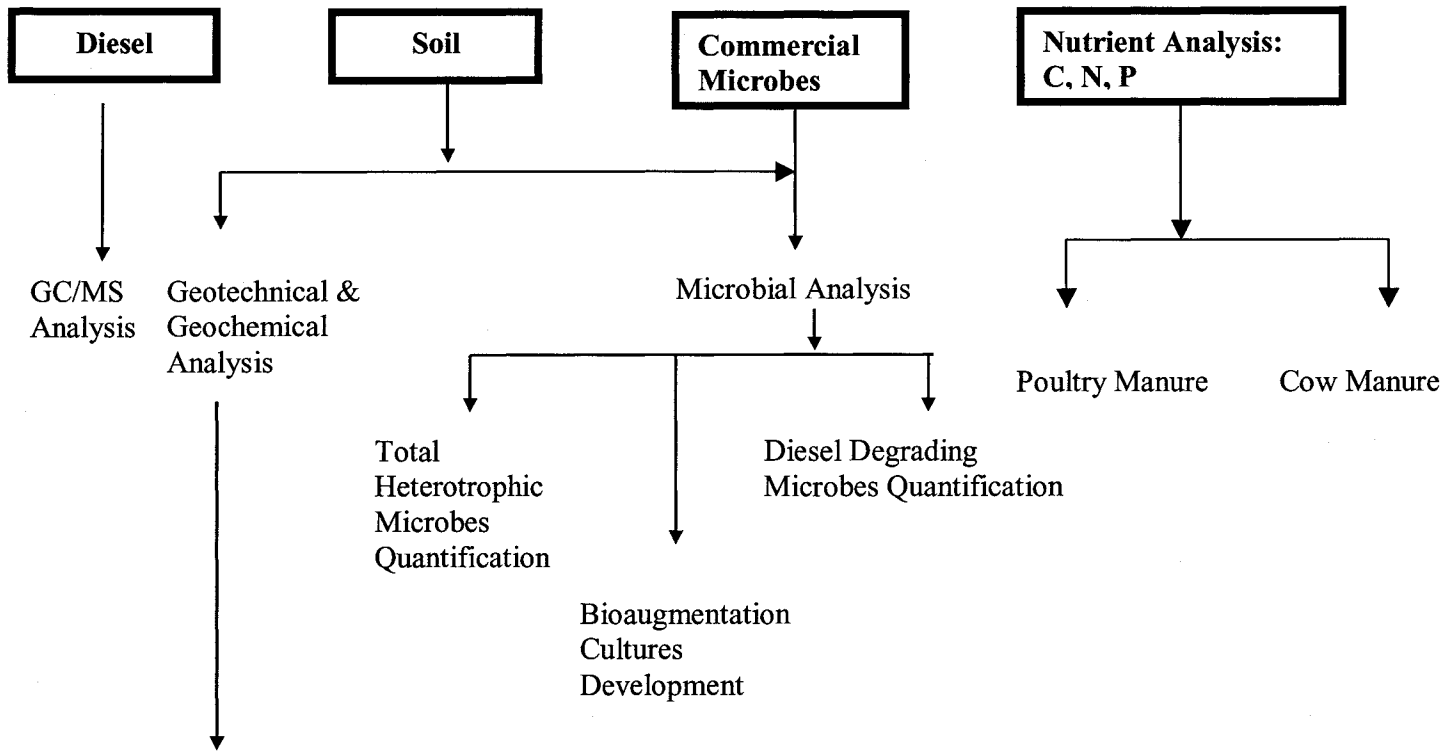


Figure 3.1: A flow chart of experimental procedure

3.3 Soil Analysis

3.3.1 Geotechnical and Geochemical Analysis of Soil

Geotechnical properties of the soil that include soil pH, soil moisture content and particle size analysis and geochemical characteristics that include soil organic carbon, total nitrogen, and total phosphorus contents were estimated for the biodegradation experiments.

3.3.1.1 Soil pH

The pH of the soil was measured using the soil pH determination method of the Analytical Method Manual of Research Branch Agriculture Canada (Sheldrick, 1984).

3.3.1.2 Soil Moisture Content

The soil moisture content was determined by the method of the Analytical Method Manual of Research Branch Agriculture Canada (Sheldrick, 1984).

3.3.1.3 Particle Size Analysis

The particle size distribution of soil was determined using the ASTM D422-63 method (ASTM 2002).

3.3.1.4 Organic Carbon Estimation

The organic carbon content of the soil was estimated using the Wakley-Blackey method of the Analytical Method Manual of Research Branch Agriculture Canada (Sheldrick, 1984).

3.3.1.5 Total Nitrogen Determination

The nitrogen content of the soil was determined according to the Kjeldahl method involving the use of a Block Digestor 2020 and a steam distillation system (Kjeltec 1002) with application note-AN 300 of Tecator Co. 1996 operation manual.

3.3.1.6 Total Phosphorus Determination

The amount of total phosphorus available in the soil was determined using the Vanadomolbdophosphoric acid colorimetric method of Olsen and Sommers, (1982).

3.3.1.7 Determination of Potassium, Magnesium, Calcium and Iron Contents

The potassium, magnesium, calcium and iron contents of the soil were evaluated by EPA methods 7610, 7450, 7140 and 7380 respectively (USEPA, 1986).

Analysis of the soil showed that it was a sandy loam soil with the characteristics given in Table 3.3. The absence of clay in the soil facilitated the biodegradation experiments as indicated in Table 3.3.

Table 3.3 Geotechnical and Geochemical Properties of Mixed Soil

Parameters	Value
pH	4.1
Moisture Content (%)	48.3
Organic Carbon (%)	2.14
N (%)	0.125
P (%)	0.0375
K (%)	0.1
Mg (%)	0.2
Ca (%)	0.1
Fe (%)	2.4
Particle Size Distribution:	
Sand (%)	85.8
Clay (%)	0
Silt (%)	14.2

3.3.2 Microbial Analysis of Soil and Commercial Microbial Samples

Prior to the enumeration of the total heterotrophic microorganisms and the diesel degrading microorganisms in the soil sample and commercial microbial samples, a suspension of each sample was prepared as follows; one gram each of the soil and commercial microbial samples were added to 9 ml and 45 ml of 0.85% NaCl (saline solution) respectively and vortexed vigorously. One ml of the suspension obtained from the commercial microbial sample prepared above was taken and added to another 9 ml of saline solution for serial dilutions. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of both the soil sample and commercial microbial samples were made.

Total heterotrophic microorganisms in each sample were quantified by the spread plate technique on a solid organic medium of trypticase soya agar (TSA, Difco), which is a rich complex medium designed for growing most chemoorganotrophic bacteria.

TSA (40 g) were added to distilled water and mixed thoroughly. Sterilization of the TSA medium was carried out in an autoclave at 121°C and 15 psi for 30 minutes. 15 ml of the medium (TSA) was plated into 10 cm diameter disposable petri dishes and the plates were inverted after solidification and allowed to dry at 25°C overnight. Plate count was performed as follows: Two replicates of 0.1 ml aliquots of each of the serial dilutions of 10^{-1} to 10^{-5} of the soil and microbial product suspension were then plated with the above medium. After spreading, the plates were inverted and allowed to culture at 37°C for 24 hours in an incubator. Colonies were then counted using a Quebec colony counter.

Quantification of diesel degrading microorganism in both the soil and commercial microbial product supplied by Universal Environmental Services was done by the spread plate technique. A synthetic medium in which commercial diesel-fuel was the sole carbon source was used. The composition of the medium was 0.13% NH_4NO_3, 0.05% $MgSO_4.7H_2O$, 0.02% $CaCl_2.2H_2O$, 0.5% KH_2PO_4, 0.5% K_2HPO_4 and 1.5% Agar. Sterilization of the synthetic medium was carried out in an autoclave at 121°C and 15 psi for 30 minutes, before the addition of 0.2% diesel fuel. Fifteen ml of the synthetic medium was plated into 10 cm diameter disposable petri dishes and the plates were inverted after solidification and allowed to dry at 25°C overnight. Plate count was performed as follows: Two replicates of 0.1 ml aliquots of each of the serial dilutions of 10^{-1} to 10^{-5} of the soil and microbial product suspension were then plated with the synthetic medium. After spreading, the plates were inverted and inoculated at 37°C for 72 hours in an incubator. Colonies were then counted using a colony counter. The results obtained are summarized in Table 3.4.

It was observed that the number of diesel degrading microbes in the mixed soil outnumbered those in the commercial microbial sample, since the mixed soil contained diesel-contaminated soil.

Table 3.4 Microbial Properties of Soil and Commercial Microbial Sample

	Soil Sample (cfu/g)	**Commercial Microbial Sample (cfu/g)**
Total Heterotrophic Microbes	1.54×10^5	7.25×10^7
Diesel Degrading Microbes	5.5×10^5	3×10^4

Calculation of the colony forming units per gram of soil or per ml (cfu/g or cfu/ml)

$$No\ of\ cfu/g\ of\ soil = \frac{Average\ number\ of\ colony \times Dilution\ factor}{ml\ transferred\ to\ plate}$$

3.4 Diesel-fuel Characterization

Prior to the bioremediation experiments, the commercial diesel fuel was characterized in order to obtain the gas chromatogram of diesel fuel alone.

3.4.1 Gas Chromatography Analysis of Commercial Diesel-Fuel

The analysis of the commercial diesel-fuel was performed with a Hewlett-Packard (U.S.A) 5890 series Gas Chromatograph (GC) coupled to a 5970 series mass selective detector (MSD) which was controlled by a Hewlett-Packed personal computer operated by HP MS ChemStation version B.02.05 software using Microsoft Windows 3.1. The column used was a DB-5 capillary column (30 m length, 0.25 mm ID, 0.25 mm film

thickness and cross linked with 5% phenylmethyl silicone) in a splitless injection mode. The carrier gas was helium (He) and the column head pressure was 12 psi, while the injection volume was 1 µl. The initial oven temperature of 40°C was maintained for 4 minutes, the temperature was raised at 10°C/min and the final temperature of 270°C was held for 5 minutes. The injector and detector were set at 300°C and 280°C respectively. Commercial diesel-fuel was diluted in hexane prior to gas chromatography analysis. Diesel fuel (10 mg) was diluted with 1 ml of hexane giving a concentration of 10 mg/ml in hexane. The identification of the individual compounds was performed using a library search, and the software used for the library search was the AMDIS (Automated Mass Spectral Deconvolution and Identification System) coupled to the NIST/EPA/NIH Mass Spectral Library Search (NIST v.2.0). The gas chromatogram of the commercial diesel fuel is in Appendix A: (A.1).

3.5 Laboratory Evaluation of Bioremediation Potential of Soil and Commercial Microbes.

The assessment of biodegradation potential can be performed by a laboratory treatability study or extensive waste characterization combined with the simulation of bioremediation potential based on biodegradability data for a given type of compound (Gogoi et al., 2003). In this section, the indigenous diesel degrading microorganisms from the soil and the commercial microbes were evaluated for their efficacy in the biodegradation of diesel fuel from the laboratory shake flask experiments and the plate inoculating technique.

3.5.1 Laboratory Shake Flask Experiments

The laboratory shake flask experiment is an enrichment technique, which was carried in a mineral salt solution (MSS) and 1ml of a trace element solution. The composition of the MSS used in the shake flask experiments is given in Table 3.5.

Table 3.5 Composition of mineral salt solution with a medium pH of 7.0

Name of Salt	**Amount in g/l**	**Composition of trace element solution**	
K_2HPO_4	4.74	**Name of Salt**	**Amount in g/l**
KH_2PO_4	0.56	$CuSO_4.5H_20$	10
$MgSO_4.7H_2O$	0.5	H_3BO_3	10
NaCl	1	$MnSO_4.5H_20$	10
$FeCl_3$	0.01	$ZnSO_4.7H_2O$	70
$CaCl_2.2H_2O$	0.01	MOO_3	10
NH_4NO_3	2.5		

The shake flask experiments were carried out in 125 ml flasks incubated at 30°C in a Gyrotory Water Bath Shaker (Model G76D), manufactured by New Brunswick Scientific Co. Inc., Edison, N.J., USA at 170 revolutions per minutes (rpm) for two weeks. The degradation capacity of the indigenous microorganisms was assessed qualitatively from the virtual observation of growth and colour change of the growth medium and also by the measurement of the optical density of cell suspensions in each flask in a UV Spectrophotometer at 600 nm.

Each experimental flask for the soil contained 50 ml MSS; that was autoclaved at 121°C and 15 psi for 30 minutes, one ml of the suspension of the saline physiological

solution containing the soil indigenous microorganisms and diesel fuel at 1% (v/v) which was added as the sole carbon source. The experimental blank contained 50 ml MSS and 1% (v/v) diesel fuel in a 125 ml flask.

A control experiment involved a 125 ml flask containing 50 ml MSS; sterilized by autoclaving, one ml of the suspension of the saline physiological solution containing the soil indigenous microorganisms, but with no diesel. The control blank contained 50 ml MSS only. The experimental and control blanks were used to re-set the UV Spectrophotometer reading back to zero before measuring optical density of the contents of each flask. All experiments were incubated for two weeks after which the absorbance change or optical density of the mineral media was measured in a UV Spectrophotometer at 600 nm. The same experimental procedure was used for the commercial microbial sample to establish their biodegradative ability in the degradation of diesel fuel. A simple illustration of the shake flask experiment is shown in Figure 3.2.

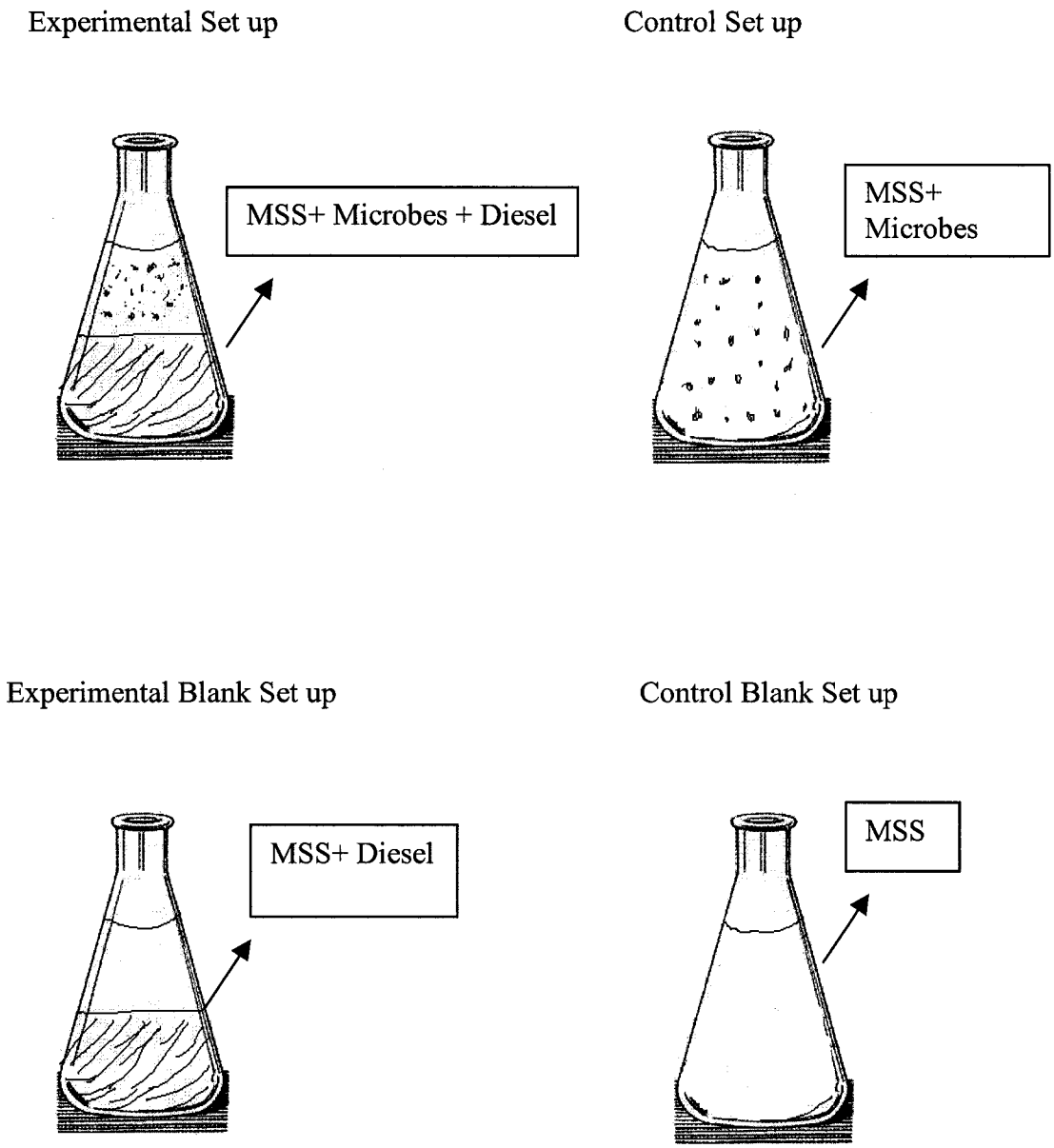


Figure 3.2: A simple illustration of the laboratory shake flask experiments

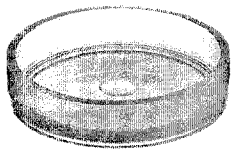
3.5.2 Plate Inoculating Technique

The plate inoculating technique was carried using the procedure of Gallego et al. (2001) on two types of growth media, namely; the TSA and Synthetic media. The composition of the synthetic medium was 0.2% diesel fuel as carbon source (v/v), 1% (v/v) bactor agar as solidifying medium and a mineral salt solution of 0.13% NH_4NO_3, 0.05%$MgSO_4.7H_2O$, 0.02% $CaCl_2.2H_2O$, 0.5% KH_2PO_4 and 0.5%K_2HPO_4.

Soil (1 g) was added to 9 ml of 0.85% (v/v) NaCl to isolate the soil indigenous microorganisms from soil and the suspension was vortexed vigorously. Aliquots (0.1ml) of the suspension was spread plated first on the synthetic agar plates and incubated for 72 hours at 30°C. The same procedure was used for the commercial microbial sample, except a suspension of the commercial microbial sample was prepared from the powder form in which 1 g of the sample was added to 45 ml of 0.85% (v/v) NaCl.

After 72 hours at 30°C, the cells were inoculated onto fresh TSA plates. The transferred cells had become induced diesel degraders. Diesel fuel (0.5 ml) was pipetted into disposable cultures tubes, to provide a carbon source in vapour form. The disposable culture tubes were plugged with cotton and were placed on the lids of the TSA plates, which were inverted and incubated for 24 hours at 30°C. The diesel allowed the induction of enzymes(s) involved in the biodegradation of this substrate, which resulted in an increase in the number of cells for both the soil and commercial microbial samples. A simple illustration of the plate inoculating technique is shown in Figure 3.3.

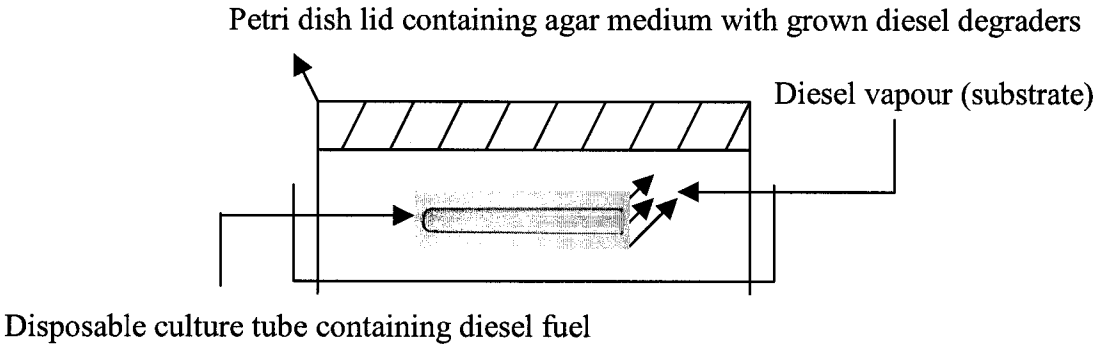
(i) Synthetic medium agar plates containing microbes



(ii) Incubation for 72 hours at 30°C

↓

(iii) Transfer to TSA medium plates with disposable culture tubes containing diesel fuel



↓

(iv) Incubation for 24 hours at 30°C

Figure 3.3: A simple illustration of the plate inoculating technique

Chapter 4

Bioremediation Experimental Design

The biodegradation experiments were conducted in two phases. Phase I experiments determined the effect of one type of nutrient (either poultry manure or liquid cow manure), one type of inoculum (either indigenous or exogenous microbial inoculum) and one type of bulking agent (either sand or hay) on the degradation of diesel fuel in soil. Phase II experiments involved a series of laboratory based experiments conducted to study the interactions among the nutrients, inocula and bulking agents using different combinations.

A 2^3 full factorial design with 2 levels for the phase II experiments generated by Design-Expert® version 6 software for Design of Experiments (DOE), by Stat-Ease, Inc. MN., USA was used. (Montgomery, 2001; Lye, 2003). The variable of response in phase I and II experiments was the TPH content, expressed as % degradation. The % biodegradation was analyzed to determine the most significant factors and any interactions using the software.

4.1 Experimental Procedure

A flow chart of the experimental procedure employed for the bioremediation experimental design is depicted in Figure 4.1. All experiments were done in duplicate.

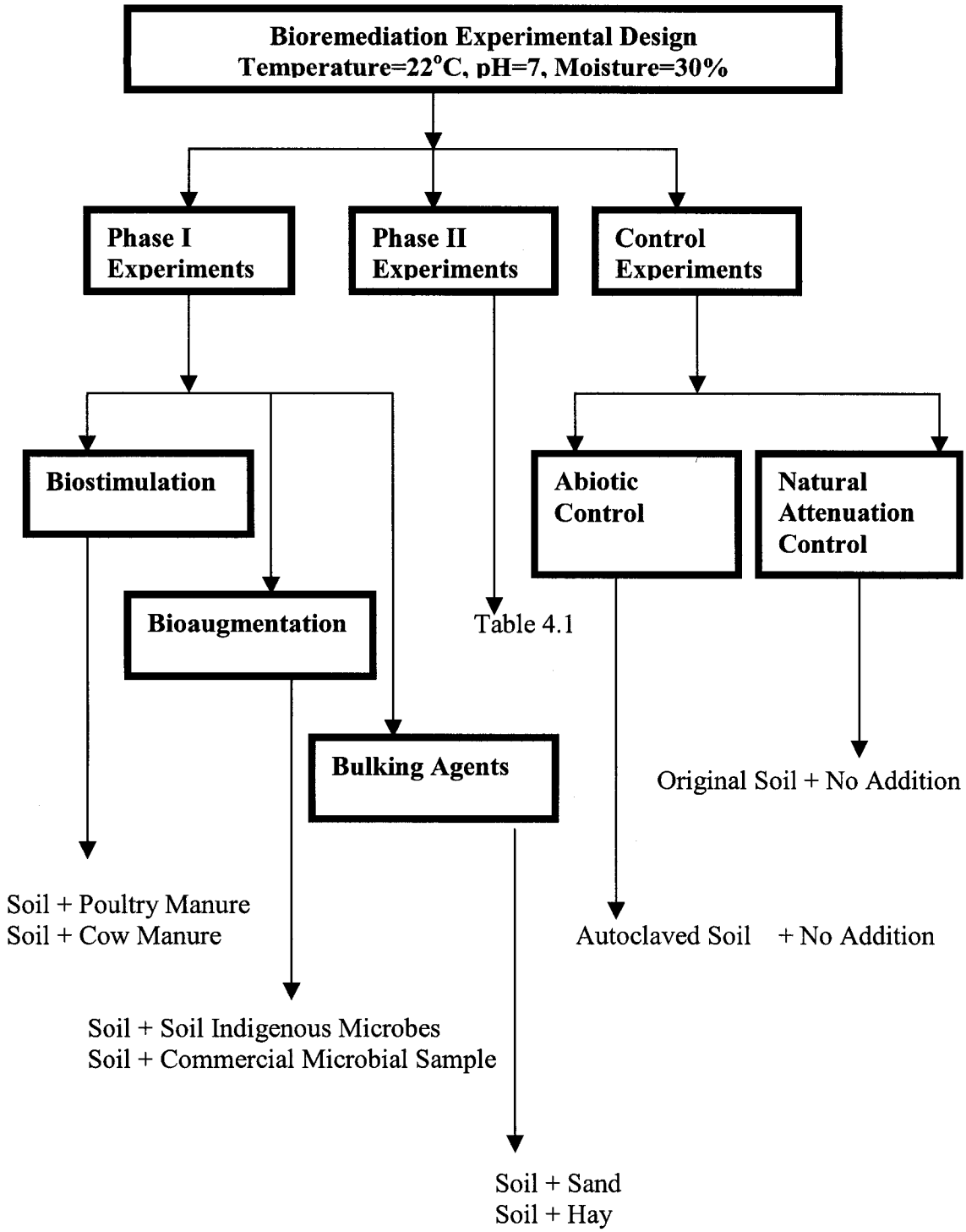


Figure 4.1: A flow chart of experimental procedure for biodegradation experiments

Table 4.1 Phase II Biodegradation Experiments
Experimental design matrix (2^3 full factorial design)

	Factor 1	**Factor 2**	**Factor 3**	**Response**
	Nutrients (mls)	Microbes (cfu/g)	Bulking Agents (grams)	% TPH Degradation
1	Poultry Manure	Soil Indigenous Microbial Inoculum	Chopped Hay	
2	Cow Manure	Soil Indigenous Microbial Inoculum	Chopped Hay	
3	Cow Manure	Commercial Microbial Sample Inoculum	Chopped Hay	
4	Cow Manure	Commercial Microbial Sample Inoculum	Sand	
5	Cow Manure	Soil Indigenous Microbial Inoculum	Sand	
6	Poultry Manure	Soil Indigenous Microbial Inoculum	Sand	
7	Poultry Manure	Commercial Microbial Sample Inoculum	Sand	
8	Poultry Manure	Commercial Microbial Sample Inoculum	Chopped Hay	

4.1.1 Inoculum Production

For the biodegradation experiments involving the application of bioaugmentation technique, the two different types of inocula employed were,

1) soil indigenous microorganisms (SIM) inoculum
2) commercial microbial sample (CMS) inoculum

The two types of inocula were prepared by suspending both the indigenous microorganisms of the mixed soil and the commercial ample microorganisms in sterile

saline. One ml each of each suspension was pipetted into 125 ml flasks containing 50 ml MSS, that was sterilized by autoclaving. Diesel fuel (1 %, v/v) and succinate (1%, w/v) were added into the flasks and the flasks were incubated on a shaker at 170 rpm for 3 days. The culture broth (50 ml each) obtained consisted of a mixed consortium of microbes. The microbes were collected by centrifugation at 10,000 rpm for 15 minutes, and washed in sterile saline three times to remove inorganic nutrients and residual diesel. The cell masses collected from each flask for both soil and commercial microbial samples were suspended in separate 75 ml saline solutions and these suspensions were used for the bioaugmentation experiments.

4.1.2 Addition of Manures to Contaminated Soils

Manures were sterilized by autoclaving to kill their microbiological content, while preserving their nutritional properties. Ten grams of each of the autoclaved cow manure and poultry manure were added to the treatment conditions where needed.

4.1.3 Addition of Bulking Agents to Contaminated Soils

Chopped hay (5 g) and 50 g of Ottawa sand were thoroughly mixed with the contaminated soil (100 g) in the experimental units where either was required.

4.1.4 Inoculum Addition to Contaminated Soils

Five ml each of the two types of inocula was added to the treatment conditions where needed. In the biodegradation experiments, the SIM and CMS inocula was added to systems where needed without autoclaving the soils, but the soils were autoclaved in the control experiments.

4.2 Description of the Experimental Systems

The biodegradation experiments in soil microcosms were carried out in 250 ml conical flasks, containing 100 grams of diesel contaminated soil and closed with plastic foam stoppers as illustrated in Figure 4.2.

The uncontaminated soil had a pH of 4.1, which was adjusted to 7.0 with 0.5 N NaOH prior to the addition of diesel fuel to soil. The soil moisture content of 48% was also adjusted to 30%. Soil (100 g) was put into a 250 ml flask and 1 g of diesel fuel was added to make a concentration of 10,000 mg diesel per kg of soil for each experimental treatment combination set up. Diesel fuel was allowed to penetrate deeply in the soil for 5-10 minutes and the flasks were shaken vigorously several times to ensure a thorough dispersion of diesel fuel in the soil matrix.

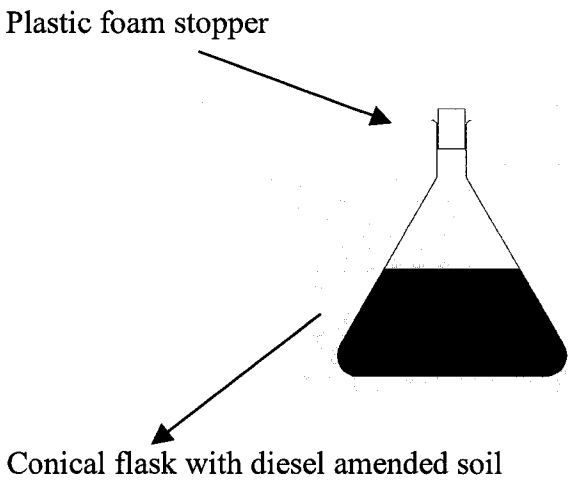


Figure 4.2: Illustration of the biodegradation experimental set up.

For oxygen incorporation into the experimental systems, plastic foam stoppers were utilized. The soil moisture content was maintained at 30% by weighing the experimental flasks every 2 days and aseptically adding deionised water to replace any water losses. A total of 32 experiments including duplicates were conducted at a room temperature of 22°C for a 90-day period. The treatment combinations for the phase I, control experiments and the phase II experiments are depicted in Tables 4.3 and 4.4

Table 4.2 Phase I and Control Experiments to Determine Individual Effects of Nutrients, Microorganisms and Bulking Agents.

		Nutrients		Microorganisms		Bulking Agents	
Treatments	**Diesel Amended Soil (g)**	**Poultry Manure (g)**	**Cow Manure (g)**	**Soil Indigenous Microbial Inoculum (mls)**	**Commercial Microbial Sample Inoculum (mls)**	**Sand (g)**	**Hay (g)**
1 and 2	100	-	-	-	-	50	-
3 and 4	100	-	-	-	-	-	5
5 and 6	100	-	-	-	5	-	-
7 and 8	100	-	-	5	-	-	-
9 and 10	100	10	-	-	-	-	-
11 and 12	100	-	10	-	-	-	-
13 and 14 AbioticControls A and B	100 (Autoclaved)	-	-	-	-	-	-
15 and 16 Natural Attenuation Controls A and B	100	-	-	-	-	-	-

Table 4.3 Phase II Experiments with Nutrient, Microorganism and Bulking Agent Combinations.

		Nutrients		Microorganisms		Bulking Agents	
Treatment Combination	**Diesel Amended Soil (g)**	**Poultry Manure (g)**	**Cow Manure (g)**	**Soil Indigenous Microbial Inoculum (mls)**	**Commercial Microbial Sample Inoculum (mls)**	**Sand (g)**	**Hay (g)**
17 and 18	100	10	-	5	-	-	5
19 and 20	100	-	10	5	-	-	5
21 and 22	100	-	10	-	5	-	5
23 and 24	100	-	10	-	5	50	-
25 and 26	100	-	10	5	-	50	-
27 and 28	100	10	-	5	-	50	-
29 and 30	100	10	-	-	5	50	-
31 and 32	100	10	-	-	5	-	5

4.3 Quantitative Determination of Total Petroleum Hydrocarbon Concentration (TPH)

The biodegradation experiments were monitored at 45 days and 90 days by sampling the contents of the flasks to estimate the TPH concentration in mg/kg and the results are expressed as percent (%) degradation. The analysis of TPH concentration involved three stages; these are the extraction of contaminated soil, the clean-up of soil extracts and the GC analysis of cleaned extracts.

4.3.1 Extraction of Petroleum Hydrocarbons from Soils

The petroleum hydrocarbons in the soil were extracted by soxhlet extraction using EPA method 3541 and Automated Soxhlet Extractor, the Soxtec HT-2 extraction system, with temperature-controlled oil bath (Tecator Co., Sweden).

4.3.2 Silica Gel Clean-up of Extracted-Hydrocarbons

The soil extracts were cleaned prior to GC analysis using the general guidance of USEPA method 3600C. The clean up procedure was performed using an activated silica gel (one gram, 60-120 mesh) which was packed into a Pasteur pipette and clamped unto a retort stand. The soil extract (1 ml - final volume) was added drop wisely and washed unto the silica-packed Pasteur pipette and 5 ml of the eluate was collected in a 15 ml graduate conical cylinder. The cleaned extracts were transferred to a 5 ml and 2 ml vials for storage at 4°C in a refrigerator until GC analysis could be conducted.

4.3.3 Gas Chromatograph Procedures

A gas chromatograph with a flame ionization detector (GC/FID) was used, as it has been proven to be ideal for the quantification of total petroleum hydrocarbons (Douglas et al., 1992). The equipment used to perform TPH analysis using the Tier 1 and Tier 2 Petroleum Hydrocarbon Methods (Atlantic RBCA, 1999) in conjunction with EPA method 8015B (USEPA 1986a) by Maxxam Analytics Inc., St. John's, Newfoundland was an Agilent 6890 Series model G1530A GC system with Agilent Flame Ionization Detector and they were controlled by a Pentium III computer (1GB) operated by Agilent GC ChemStation (Rev.A.08.03 (847) software using Microsoft Windows NT version 4.00.1381. TPH samples were automatically injected into the GC/FID by Agilent Auto Sampler, 7683 series injector, model G2613A.

The instrument conditions were as follows;

Column:	Rtx-5 sil MS, Dual 7.5 m × 32 mm × 1.0 μm film
Carrier gas:	Helium carrier gas (1.5 ml/min at 250°C)
Injector:	250°C, splitless, split on at 0.5 min, off at 13.0 min.
Detector:	FID, 300°C, Air 450 ml/min, Hydrogen 30 ml/min.
Oven Program:	55 °C, no hold
	50 °C/min to 95 °C, no hold
	20 °C/min to 150 °C, no hold
	60 °C/min to 290 °C, hold 7.5 min
	Run Time = 13.38 min
Injection volume:	2 μl

4.3.3.1 Calibration Standards and Curves

A calibration standard is a sample containing a known amount of the compound to be quantified. A calibration curve is a graphical presentation of the amount and response data obtained from two or more calibration samples or standards. In a GC/FID procedure, a known amount of calibration standard is injected and a chromatogram is obtained, and the response (the area beneath the peaks) calculated. To obtain reliable quantitative results, a calibration curve is usually based upon at least three calibration standards, which bracket the concentrations expected to be found in the unknown sample.

The calibration table prepared by Maxxam Analytics Inc., St. John's, Newfoundland, which is in Appendix A (A.2) consisted of mixed compound standards of known concentration. Two sets of different mixtures were used; these are the Fueloil range mixture and the Lubeoil range mixture.

The Fueloil range calibration standard consisted of six compound standards that included; Napthalene, Dodecane (C_{12}), Acenaphthene, Hexadecane (C_{16}), Anthracene and Heneicosane (C_{21}) and which were used to calibrate the Fueloil range ($> C_{10} - < C_{21}$). The Fueloil range calibration curve given in Appendix A (A.3) was made on six calibration levels. The Lubeoil range calibration standard consisted of three compounds standards that included; Crysene, Octacosane (C_{28}) and Benzo(A)pyrene that were used to calibrate the Lubeoil range ($> C_{21} - < C_{32}$) and the Lubeoil range calibration curve given in Appendix A (A.4) was made on six calibration levels. The gas chromatogram containing the compounds used for the Fueloil range and Lubeoil range is given in Appendix A (A.6). The calibration curve obtained for the surrogate compound; O-Terphenyl, used in

monitoring extraction efficiency is given in Appendix A (A.5). A regression coefficient (R) of 0.99 or 1 is required for the quantification of hydrocarbons in environmental matrixes by USEPA 8015B method.

The % TPH degradation was calculated as shown below from the response (area beneath the peaks) of the chromatograms obtained from the GC/FID analysis report:

$$\% \text{ TPH Degradation} = \frac{\text{Initial TPH Concentration - Final TPH Concentration}}{\text{Initial TPH Concentration}} X\ 100$$

Where;

Initial TPH Concentration = 10,000 mg/kg

The final TPH Concentration was obtained from the addition of the concentration of the Fueloil range (µg/ml) and the concentration of the Lubeoil range (µg/ml) from the gas chromatograms obtained for all treatment systems in phase I and phase II experiments after 45 days and 90 days.

4.3.3.2 Calculation of TPH concentration in soil

The concentration of diesel fuel TPH (mg/kg) in a soil sample after GC/FID analysis was calculated as follows:

$$\text{TPH (µg/ml or mg/kg)} = \frac{\text{C (µg/ml)}}{\text{W (g)}} X\ \text{V (ml)}$$

Where:

C = concentration in TPH obtained from the calibration curve of the GC/FID analysis

W = weight of dry soil = 5 g

V= final volume of extract = 5 ml

4.4 Statistical Analysis of Results

All statistical analyses of phase II experiments were performed using the 2^3 full factorial design with 2 levels of the Design-Expert® statistical software employed in the design of experiments-DOE (Lye, 2003; Montgomery, 2001). To evaluate the effect on % degradation and method performance of the 3 variables (nutrients, microorganisms and bulking agents), the % degradation data were analyzed by analysis of variance (ANOVA). A 5% significance level was used to determine whether a factor or an interaction was statistically significant.

Chapter 5

Results and Discussion

5.1 Laboratory Shake Flask Experiments

The results obtained for the laboratory shake flask experiments for the soil and commercial samples are depicted in Table 5.1 and Table 5.2 respectively.

Table 5.1 Results of the Laboratory Shake Flask Experiments for Soil Indigenous Microbes (SIM)

Experimental			Control		
Design	**Initial OD**	**Final OD**	**Design**	**Initial OD**	**Final OD**
MSS + DF	0.00	0.00	MSS	0.00	0.00
MSS + DF + SIM I	1.081	1.924	MSS + SIM I	1.070	1.036
MSS + DF + SIM II	1.076	1.916	MSS + SIM II	1.070	1.040

Table 5.2 Results of the Laboratory Shake Flask Experiments for Commercial Microbial Sample (CMS)

Experimental			Control		
Design	**Initial OD[1]**	**Final OD**	**Design**	**Initial OD**	**Final OD**
MSS[2] + DF[3]	0.00	0.00	MSS	0.00	0.00
MSS + DF + CMS I	0.448	-0.699	MSS + CMS I	0.133	0.145
MSS + DF + CMS II	0.617	-0.524	MSS + CMS II	0.135	0.140

[3]DF = diesel fuel; [1]OD = Optical Density; [2]MSS = Mineral Salt Solution.

The changes in the optical density and in colour of the enriched culture medium of the experimental flasks containing the SIM when compared to their control flasks indicated their ability to degrade diesel hydrocarbons. Moreover, the increase in the optical density signified that the cells used diesel fuel as a carbon source. However, negative values of optical density and no observed colour change obtained from the experimental flasks containing the CMS when compared to their control flasks showed that the CMS microorganisms were being killed with 1% (v/v) diesel in these liquid medium tests as indicated by the decrease in the optical density readings obtained, hence, no increase in the cell numbers of the CMS microorganisms.

5.2 Plate Inoculating Technique

When the TSA plates were viewed under a microscope, both soil and commercial microbial sample microorganisms grew well when inoculated onto TSA plates after their initial growth on synthetic agar plates, which contained diesel vapours. The microbes effectively utilized diesel fuel vapour and it could be inferred that they possess the capability to degrade diesel fuel. No data could be given to support this result, because the enumeration of cells from the TSA plates could not be carried out because the cells were too numerous to count.

5.3 Biodegradation Experiments

Initially soil was contaminated with a TPH concentration of 10,000 mg/kg. The % TPH degradation of all treatment combinations was determined after 45 days and 90 days. Biodegradation of the diesel fuel in soil was observed in all treatment conditions.

5.3.1 Phase I Experiments

In phase I experiments, treatment with bulking agents was considered to be the most successful compared when compared to bioaugmentation and biostimulation treatments in terms of the overall time required for treatment. The addition of sand to diesel-contaminated soil yielded the highest % degradation as shown in Figure 5.1.

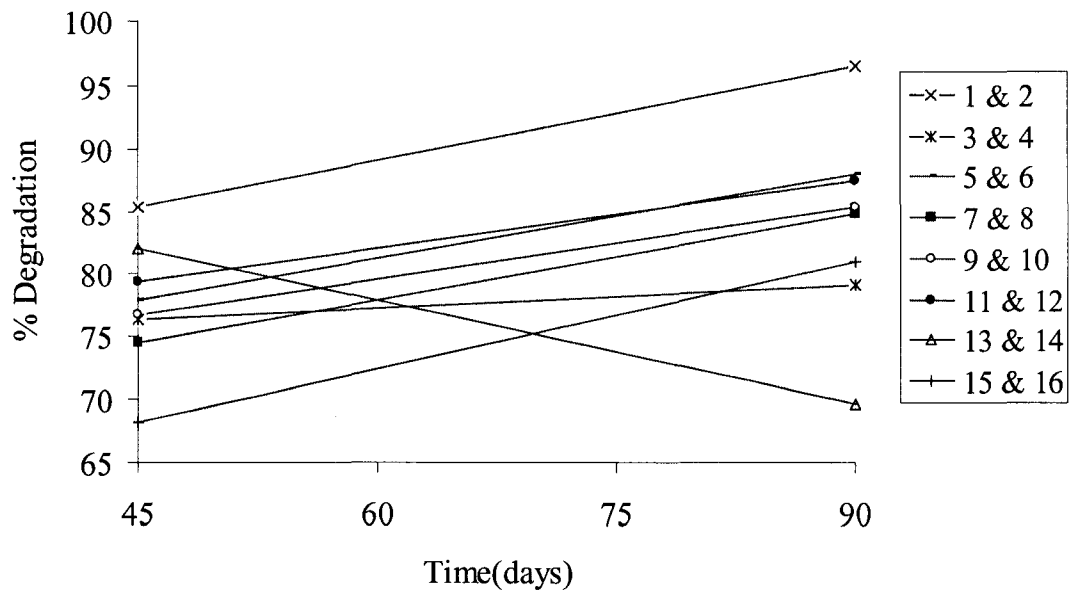


Figure 5.1: TPH analysis curve fittings for phase I experiments.

The gas chromatograms obtained all the treatment systems in phase I experiments after 45 days and 90 days are depicted in the Appendix B. The % TPH degradation of these experiments is given in Tables 5.3 and Table 5.4.

The % TPH degradation was calculated from the GC/FID analysis report obtained from the chromatograms that are shown are in Appendix B.

$$\% \text{ TPH Degradation} = \frac{\text{Initial TPH Concentration - Final TPH Concentration}}{\text{Initial TPH Concentration}} X\,100$$

Where;

Initial TPH Concentration = 10,000 mg/kg

Final TPH Concentration = Concentration obtained from addition of the Fueloil range (μg/ml) and the concentration of the Lubeoil range (μg/ml) from the gas chromatograms.

Table 5.3 Percentage Degradation of Diesel-Fuel in Phase I Experiments After 45 days.

Treatment Combinations	**Content of Treatment Systems**	**% Degradation**	**Average % Degradation**	**Figure Title In Appendix B**
1 and 2	Sand	85.4/85.3	85.4	B1, B2
3 and 4	Hay	82.0/70.5	76.3	B3, B4
5 and 6	CMS Inoculum	85.2/70.5	77.9	B5, B6
7 and 8	SIM Inoculum	71.4/77.6	74.5	B7, B8
9 and 10	Poultry Manure	82.7/70.8	76.8	B9, B10
11 and 12	Cow Manure	81.0/77.7	79.4	B11, B12
13 and 14	Abiotic Controls	89.7/74.2	82.0	B13, B14
15 and 16	Natural Attenuation Controls	65.2/71.2	68.2	B15, B16

Table 5.4 Percentage Degradation of Diesel-Fuel in Phase I Experiments After 90 days.

Treatment Combinations	Content of Treatment Systems	% Degradation	Average % Degradation	Figure Title In Appendix B
1 and 2	Sand	96.1/97.1	96.6	B17, B18
3 and 4	Hay	78.7/79.4	79.1	B19, B20
5 and 6	CMS Inoculum	86.7/89.2	88.0	B21, B22
7 and 8	SIM Inoculum	85.7/83.8	84.8	B23, B24
9 and 10	Poultry Manure	83.8/87.0	85.4	B25, B26
11 and 12	Cow Manure	89.1/85.7	87.4	B27, B28
13 and 14	Abiotic Controls	64.8/74.3	69.6	B29, B30
15 and 16	Natural Attenuation Controls	75.7/86.3	81.0	B31, B32

5.3.1.1 TPH reduction due to biostimulation

Using biostimulation alone, after 45 days, contamination had reduced in the treatment condition where cow manure was used as nutrient addition (11/12) by 79.4% from 10,000 mg/kg to 2062.4 mg/kg and in treatment system (9/10) where poultry manure was used, a reduction of 76.4% or from 10,000 mg/kg to 2323.15 mg/kg. In 90 days, the cow manure treatment systems had an overall TPH reduction of 87.4% with a final concentration of 1260.8 mg/kg while the poultry manure treatment systems had a decline of 85.4% and a final TPH concentration of 1461.1 mg/kg. This depicted that biostimulation with cow manure was better than biostimulation with the poultry manure.

The nutrients amendments by the addition of cow and poultry manures resulted in C:N:P ratios of 100:1.2:0.75 and 100:7.9:0.64 respectively. Vidali (2001) and Riser-Roberts (1998) recommend a C:N:P ratio of 100:10:1 for optimum hydrocarbon degradation which according to Demque et al. (1997) is the highest ratio reported in the

literature. However, the known ratio of 100:1.7:0.1 reported by Dibble and Bartha (1979) and of 100:0.4:0.04 reported by Huddleston (1979) are in better agreement with the findings in the present work. The lower proportion of nitrogen with the cow manure resulted in higher % degradation compared to the higher proportion of nitrogen of the poultry manure. Possibly excessive nitrogen in the soil may have been harmful to the microorganisms as reported by Walworth et al., (1997).

5.3.1.2 TPH reduction due to bioaugmentation

In the bioaugmented treatment systems, a decline in TPH concentration was higher with treatment systems having CMS inoculum (5/6) over treatment systems with SIM inoculum (7/8). 77.9% and 88.0% reduction was obtained with CMS inoculum in 45 days and 90 days respectively, with final concentrations of 2214.6 mg/kg and 1207.85 mg/kg respectively. Treatment combinations with the SIM inoculum had a 74.5% and a 84.8% reduction in both 45 days and 90 days respectively, with concentrations of 2549.8 mg/kg and 1522.4 mg/kg respectively. The CMS inoculum was better than the SIM inoculum in a soil environmental matrix as compared to the performance of CMS inoculum in the MSS in the laboratory flask experiments where SIM inoculum was able to utilize the diesel fuel as carbon source. It could be concluded that treatment systems (5/6) with CMS achieved a higher % degradation than treatment systems (7/8) with SIM because the introduction of the CMS into the diesel amended soil resulted in having two types of consortia, the first consortium being the soil indigenous population originally present in the soil and the second consortium being the introduced CMS grown cultures,

since an unsterilized soil was used for treatment systems where the CMS inoculum was added.

5.3.1.3 TPH reduction due to bulking agents

Considering treatment systems with bulking agents alone, a clear distinction was apparent between the Ottawa sand and hay as bulking agents. Contamination in the treatment combination (1/2) where sand was the bulking agent was reduced by 85.4% from 10,000 mg/kg to 1463.1 mg/kg in 45 days and a 76.3% reduction was achieved with hay as bulking agent from 10,000 mg/kg to 2376.45 mg/kg in 45 days in treatment condition (3/4). At the end of the 90 day experimental period, the Ottawa sand treatment system had a reduction of 96.6% reduction in TPH concentration from 10,000 mg/kg to 339.4 mg/kg, while the treatment system containing hay had a reduction of 79.1% from 10,000 mg/kg to 2091.95 mg/kg. Hence, Ottawa sand was better as a bulking agent when compared to the hay in these sets of phase I experiments. Hay contributed to the disappearance of hydrocarbons, but microbial growth observed on the hay during the experimental period may indicate that the microbial population was using the hay at this stage rather than the diesel fuel as a source of carbon. This may have led to a lower % degradation in treatment systems (3/4) where hay was used a bulking agent.

The abiotic controls (13/14), which are the autoclaved soil without addition of nutrients, microorganisms and bulking agents, had an average of 82% and 69.6% degradation after 45 days and 90 days respectively.

Percentage degradation of 82% and 69.2% were obtained after 45 and 90 days respectively in the abiotic controls. The abiotic controls may have undergone degradation in the form of vaporization since they do not contain microorganisms for biodegradation. All ex-situ techniques must account for the losses through volatilization (Heitzer et al., 1993; Arthurs et al., 1995, Atlas et al., 1998). The higher % degradation obtained after sampling at the 45^{th} day is probably due to loss of hydrocarbon constituents during either extraction or nitrogen drying.

The natural attenuation controls (15/16), which are soil in its original state prior to the biodegradation experiments, have an average of 68.2% and 81% degradation after 45 days and 90 days respectively. The % degradation obtained in the natural attenuation controls revealed that the soil indigenous microbes degraded diesel fuel without any addition, however a higher % of biodegradation was observed in all other treatment conditions where supplement addition of a bulking agent, nutrient or inoculum was made.

Conclusions can therefore be made that the rapid removal of diesel from soil cannot be accounted for by volatilization alone as seen in the abiotic controls and that a higher % of biodegradation is achieved with the addition of supplements rather than using soil indigenous microbes alone as seen in the natural attenuation controls.

The greatest biodegradation occurred in the phase I experiments for treatment systems (1/2) containing clean sand, suggesting that the addition of sand as a bulking agent to diesel-amended soil was better than all other treatment systems involving biostimulation and bioaugmentation. All chromatograms obtained for phase I experiments are given in Appendix B.

5.3.2 Phase II Experiments

In the phase II experiments, the highest degradation occurred in treatment combination (25/26) with SIM inoculum, Ottawa sand and cow manure as shown in Figure 5.2. The gas chromatograms obtained for the best treatment systems in phase II experiments after 45 days and 90 days are in Appendix C. The % TPH degradation of these experiments is given in Tables 5.5 and Table 5.6.

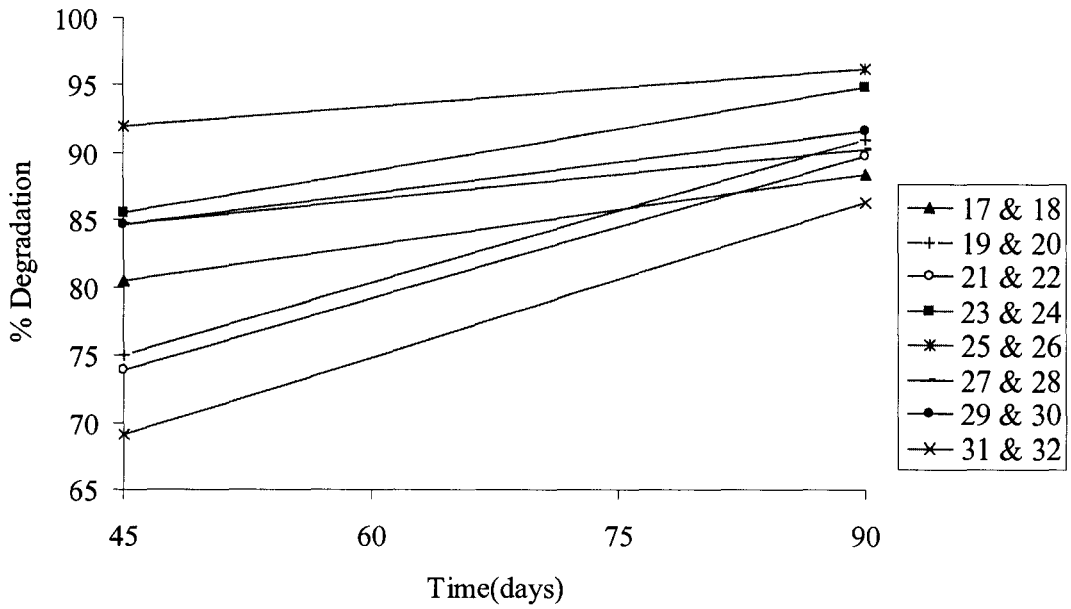


Figure 5.2: TPH analysis curve fittings for phase II experiments.

Table 5.5 Percentage Degradation of Diesel-Fuel in Phase II Experiments After 45 days.

Treatment Combinations	Content of Treatment Systems	% Degradation	Average % Degradation	Figure Title In Appendix C
17 and 18	Poultry Manure SIM Inoculum Hay	83.4/77.5	80.5	C1, C2
19 and 20	Cow Manure SIM Inoculum Hay	75.3/74.6	75.0	C3, C4
21 and 22	Cow Manure CMS Inoculum Hay	74.5/72.9	73.9	C5, C6
23 and 24	Cow Manure CMS Inoculum Sand	85.1/85.8	85.5	C7, C8
25 and 26	Cow Manure SIM Inoculum Sand	93.1/90.7	91.9	C9, C10
27 and 28	Poultry Manure SIM Inoculum Sand	84.6/86.1	84.7	C11, C12
29 and 30	Poultry Manure CMS Inoculum Sand	85.0/84.3	84.7	C13, C14
31 and 32	Poultry Manure CMS Inoculum Hay	79.8/58.3	69.1	C15, C16

Table 5.6 Percentage Degradation of Diesel-Fuel in Phase II Experiments After 90 days.

Treatment Combinations	Content of Treatment Systems	% Degradation	Average % Degradation	Figure Title In Appendix C
17 and 18	Poultry Manure SIM Inoculum Hay	88.5/88.3	88.4	C17, C18
19 and 20	Cow Manure SIM Inoculum Hay	93.0/89.0	91.0	C19, C20
21 and 22	Cow Manure CMS Inoculum Hay	89.0/90.3	89.7	C20, C22
23 and 24	Cow Manure CMS Inoculum Sand	95.2/94.3	94.8	C23, C24
25 and 26	Cow Manure SIM Inoculum Sand	98.5/93.9	96.2	C25, C26
27 and 28	Poultry Manure SIM Inoculum Sand	91.6/88.7	90.2	C27, C28
29 and 30	Poultry Manure CMS Inoculum Sand	91.4/91.8	91.6	C29, C30
31 and 32	Poultry Manure CMS Inoculum Hay	85.6/87.0	86.3	C31, C32

After 45 days, the treatment system 25/26 had the highest % degradation of 91.9% with a TPH concentration of 810.7 mg/kg. The same treatment system also had the highest rate of degradation after 90 days with a 96.2% decline in TPH concentration for which the final concentration was 373.35 mg/kg.

Other treatment conditions in phase II experiments had a noticeable decrease in the TPH concentration, such as in treatment condition (23/24) containing cow manure,

commercial microbial sample inoculum and sand, in which an average of 85.5% and 94.8% reduction was achieved in 45 days and 90 days respectively, and in close competition with treatment systems (25/26).

Though the CMS inoculum addition of treatment systems (5/6) was observed to be better than SIM inoculum addition of treatment systems (7/8) in phase I experiments, however, in phase II experiments, treatment combination (25/26) with SIM inoculum, sand and cow manure with 96.2% degradation performed better than treatment condition (23/24) with an average of 94.8% degradation, containing CMS inoculum; an inoculum that performed better than SIM inoculum in phase I experiments. This may be due to the change in the conditions of the environmental matrix in phase II experiments where the supplements additions of nutrients and bulking agents were made, which may have contributed to an increase in microbial activities and also aided in achieving a higher rate of biodegradation with treatment system containing the SIM inoculum.

Comparison of the % degradation of the best treatment conditions in phase I (1/2) and phase II experiments (25/26), suggests that these two treatment conditions are ideal for the bioremediation of diesel fuel in soil. Though the best treatment condition in phase II (25/26) had an inoculum of soil indigenous microorganisms like the best treatment system in phase I (1/2), both contained Ottawa sand as a bulking agent, but (25/26) had in addition cow manure supplement, however the average % degradation obtained was almost the same. The reason for the average % degradation obtained is due to the fact that the microbial populations in (1/2) and (25/26) responsible for diesel fuel biodegradation were the same type of microbial consortium.

The SIM inoculum addition in (1/2) and (25/26) are microbes cultured from the same source; the mixed soil for the biodegradation experiments, therefore they were already acclimatized microbes having being obtained from a soil where there is a long time spill of diesel fuel in the soil from the diesel generating plant placed on the soil and kept and watered for 4 weeks in a fish tank. Hence, the effect of the inoculum addition in the best treatment conditions for both phases I and II biodegradation experiments was insignificant. Only the sand addition played a major role in the removal rate of diesel fuel from these treatment systems; (1/2) and (25/26). All chromatograms obtained for the phase II experiments are given in Appendix C.

5.4 Statistical Analysis of Results

All statistical analyses of the phase II experiments were performed using the 2-level full factorial design of the Design-Expert® statistical software. To evaluate the effect of the addition of nutrients, inocula and bulking agents, analysis of variance (ANOVA) was undertaken using a 5% significance level to indicate statistical significance.

The addition of nutrients (A) and bulking agents (C) were statistically significant, while the addition of inocula (B) and the interactions among the nutrients, inocula and bulking agents were not statistically significant. The results are given in Table 5.7, where the F-value is the amount of variance associated with the different treatments compared with the amount of random variance. The tabulated F-value illustrates the statistical

significance of each addition at the probability (*p*) value of 0.05. If the calculated F value of each addition exceeds $p = 0.05$, then there is a very high significant difference between treatments.

Table 5.7 Significant Factors (at 5% level) in the Biodegradation Experiments Using Design-Expert® Software.

Source	**F < 0.05**		**% Contribution of Factor Effects**
Experimental Design	0.0080	significant	
A-Nutrients Addition	0.0027	significant	33.5894
B-Inoculum Addition	0.3588		1.74187
C-Bulking Agents Addition	0.0012	significant	44.0531
AB	0.5609		0.676479
AC	0.3859		1.54577
BC	0.3588		1.74187
ABC	0.3331		1.94968
Pure Error			0
Correlation Total			14.7018

Analysis of Table 5.7 revealed that the experimental design matrix (2^3 full factorial design), for the phase II biodegradation experiments was significant. The inocula addition of cultured microbes was immaterial, as evident from the low contribution effects of 1.7%, while the addition of bulking agents and nutrients; 44.1% and 33.6% positive contribution effects respectively, were of importance in these experiments.

Chapter 6

Conclusions

6.1 Summary and Conclusions

A series of laboratory-scale experiments were conducted to study the bioremediation of diesel fuel contaminated soils under different treatment conditions. The effects of factors such nutrients addition using poultry manure and cow manure, inoculum addition using indigenous soil microbial inoculum and commercial sample microbial inoculum and bulking agents addition of clean Ottawa sand and hay were evaluated in two phases; phase I and phase II biodegradation experiments. The statistical analysis of the results from phase II experiments was performed to determine additions of significance in the biodegradation experiments and the percentage effects contribution of each addition.

The following conclusions can be drawn from the results obtained:

1. Removal of diesel fuel from contaminated soils was due to biodegradation and was achieved using bioremediation treatment technologies as reported in literature.

2. The highest rate of biodegradation of diesel fuel in soil can be achieved using clean (chemically inert) Ottawa sand as a bulking agent. Rhykerd (1999) reported results that support the use of bulking agents. The advantages of clean sand as bulking agent are as follows;

i.) it is relatively simple and cost effective to use as a bulking agent since it requires no labour cost when compared to mechanical tillage for aeration within the soil matrix to increase soil porosity

ii.) it would not require removal after usage, as would other bulking agents such as rubber tire chips, which are usually removed for recycling.

iii.) it is not an organic material like hay, which is a form of carbohydrate (cellulose, a polymer of glucose) and would not be used a source of energy instead of the contaminant of concern.

iv.) clean sand would have no microbial content and would not introduce competitive organisms as could occur with clean soil, used in previous studies to increase soil porosity, but may have microbial contamination.

v.) clean sand does not introduce toxic metabolites as could occur when sawdust is used.

3. Nutrients addition is necessary in the bioremediation of diesel contaminated soil. Ma (1998), Demque at al. (1997), Gallego et al. (2001) and Cunningham et al. (2000) reported results that support the efficacy of nutrients addition.

4. Addition of acclimatized soil indigenous microbial population was completely unnecessary. Demque et al. (1997) reported similar results that using acclimatized indigenous populations in diesel-contaminated soil bioremediation was undesirable.

5. Addition of commercial microbial products does not result in the best removal rate of diesel fuel from contaminated soils. Negative reports on diesel-contaminated bioremediation using commercial bioaugmentation products have been documented (Moller et al., 1995). MendozaEspinosa and Stephenson (1996), demonstrated that natural activated sludge microorganisms performed as well in grease degradation as a commercial bioaugmentation product. What is suggested by this study is that bioaugmentation, where possible should be employed using only indigenous microorganisms that could be cultured as a balanced population in the laboratory and re-applied to the soil, if the microbial population in a contaminated soil is too low for biodegradation. Cunningham et al. (2000) also suggested this.

6. Natural attenuation is not desirable for the rapid removal of hydrocarbons from soils. Hejazi (2002) has also reported this.

7. The highest removal rate of diesel fuel from the contaminated soils can be achieved using a treatment system containing cow manure, an inoculum of soil indigenous microbial population and clean Ottawa sand.

8. The addition of nutrients and bulking agents are of statically significance in the biodegradation of the diesel fuel.

6.2 Recommendations

The recommendations are as follows:

1. The results of the laboratory experiments should be applied and implemented in a field-scale study where real environmental conditions are present.
2. The research methodology or experimental design should be applied to another major contaminant of concern such as bunker C both in laboratory and field experiments.

Appendixes

The appendixes have been sectioned into three major parts, which are

Appendix A

Appendix B

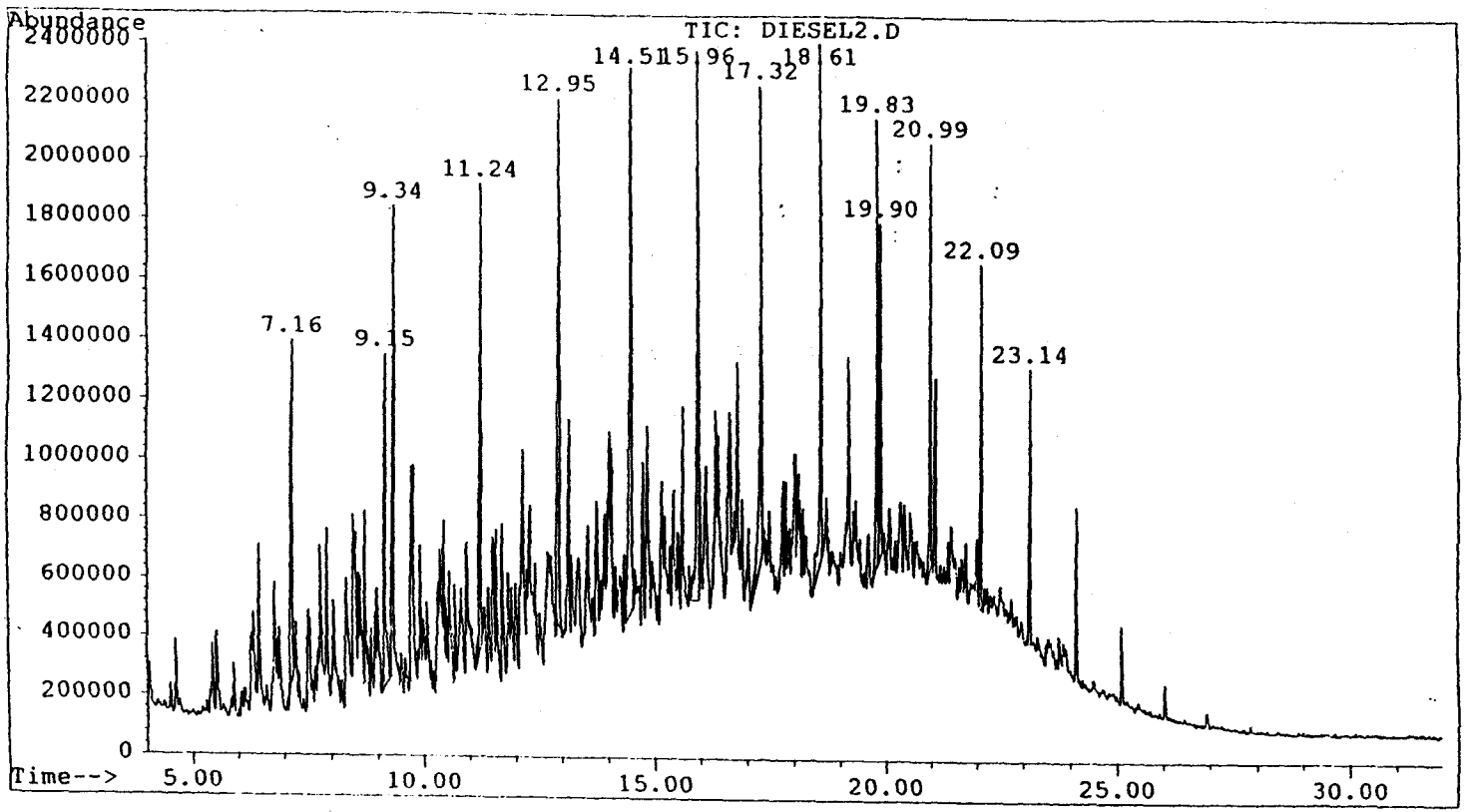
Appendix C

Appendix A

Diesel Fuel Characterization

A.1

The gas profile of the commercial diesel fuel



A.2

Calibration Table

Calibration Table

Calibration Settings for TEHS-2

Calib. Data Modified	:	05/04/14 8:26:34 PM
Calculate	:	External Standard
Based on	:	Peak Area
Rel. Reference Window	:	5.000 %
Abs. Reference Window	:	0.000 min
Rel. Non-ref. Window	:	5.000 %
Abs. Non-ref. Window	:	0.000 min
Multiplier	:	1.0000
Dilution	:	1.0000
Sample Amount	:	0.00000
Uncalibrated Peaks	:	not reported
Partial Calibration	:	Yes, identified peaks are recalibrated
Correct All Ret. Times	:	No, only for identified peaks
Curve Type	:	Linear
Origin	:	Ignored
Weight	:	Equal
Recalibration Settings:		
Average Response	:	Average all calibrations
Average Retention Time	:	Floating Average New 75%

Calibration Report Options :
Printout of recalibrations within a sequence:
Calibration Table after Recalibration
Normal Report after Recalibration
If the sequence is done with bracketing:
Results of first cycle (ending previous bracket)

Signal 1: FID2 B,

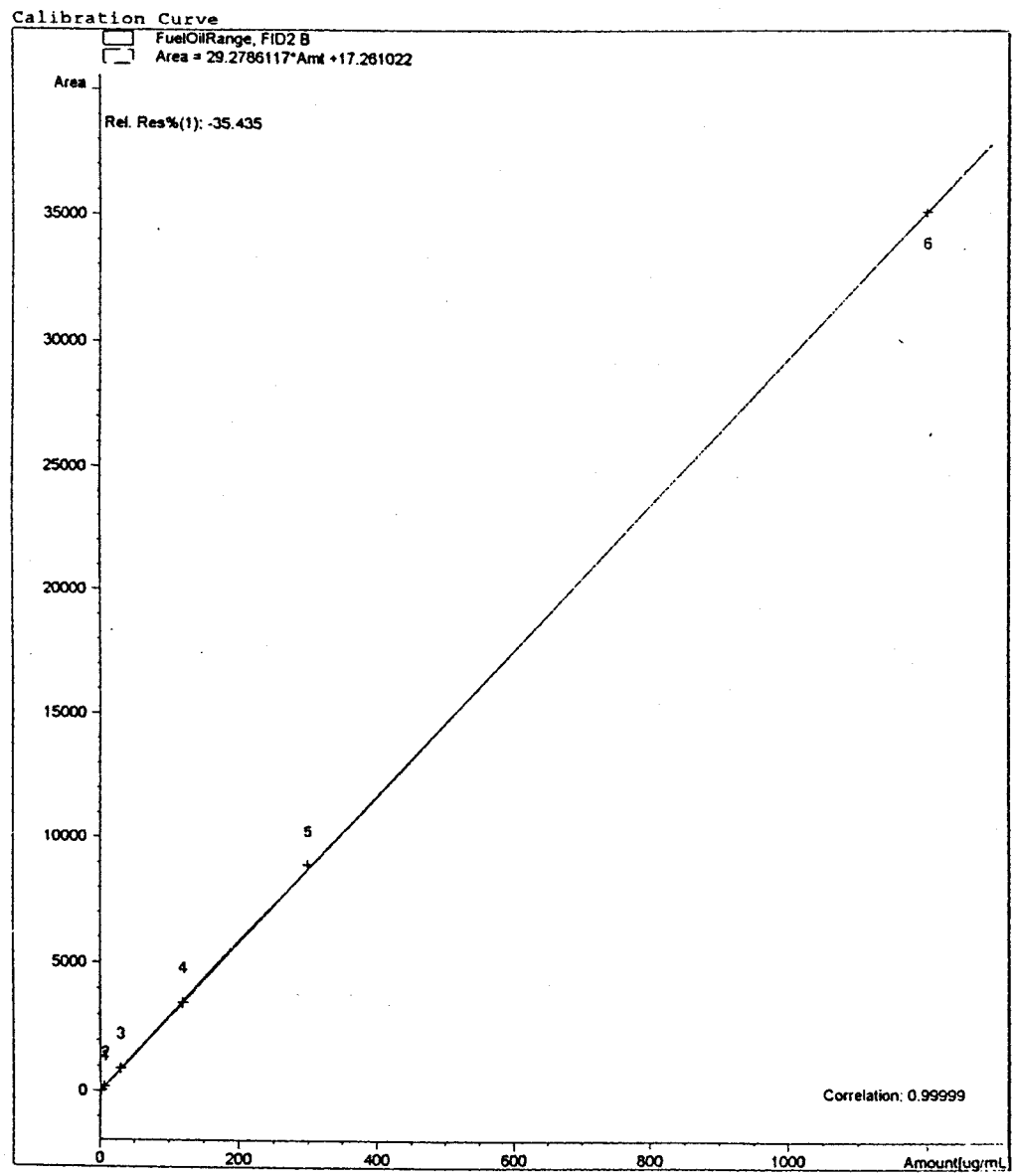
RetTime [min]	Sig	Lvl	Amount [ug/mL]	Area	Amt/Area	Ref	Grp	Name
3.907	1	1	1.50000	39.50000	3.79747e-2			FuelOilRange
		2	6.00000	179.03300	3.35134e-2			
		3	30.00000	897.50000	3.34262e-2			
		4	120.00000	3456.22700	3.47199e-2			
		5	300.00000	8935.07000	3.35756e-2			
		6	1200.00000	3.51255e4	3.41632e-2			
5.536	1	9	100.00000	1126.86157	8.87420e-2			Surrogate
7.677	1	1	7.50000e-1	20.71000	3.62144e-2			LubeOilRange
		2	3.00000	66.80500	4.49068e-2			
		3	15.00000	366.90300	4.08827e-2			
		4	60.00000	1652.86000	3.63007e-2			
		5	150.00000	3723.72700	4.02822e-2			
		6	600.00000	1.63410e4	3.67174e-2			

1 Warnings or Errors :

Warning : Curve requires more calibration points., (Surrogate)

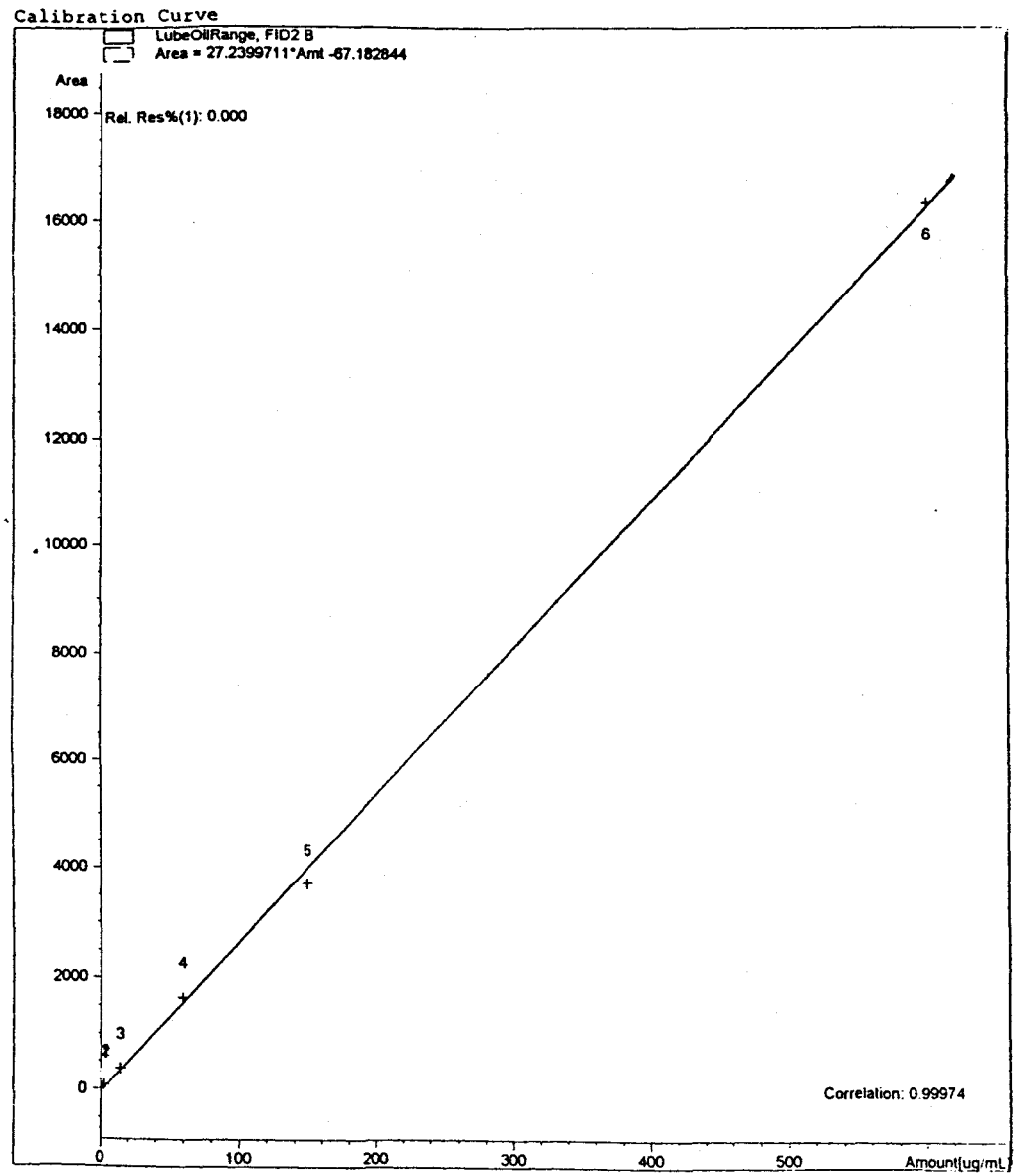
A.3

Calibration Curve of the Fueloil range



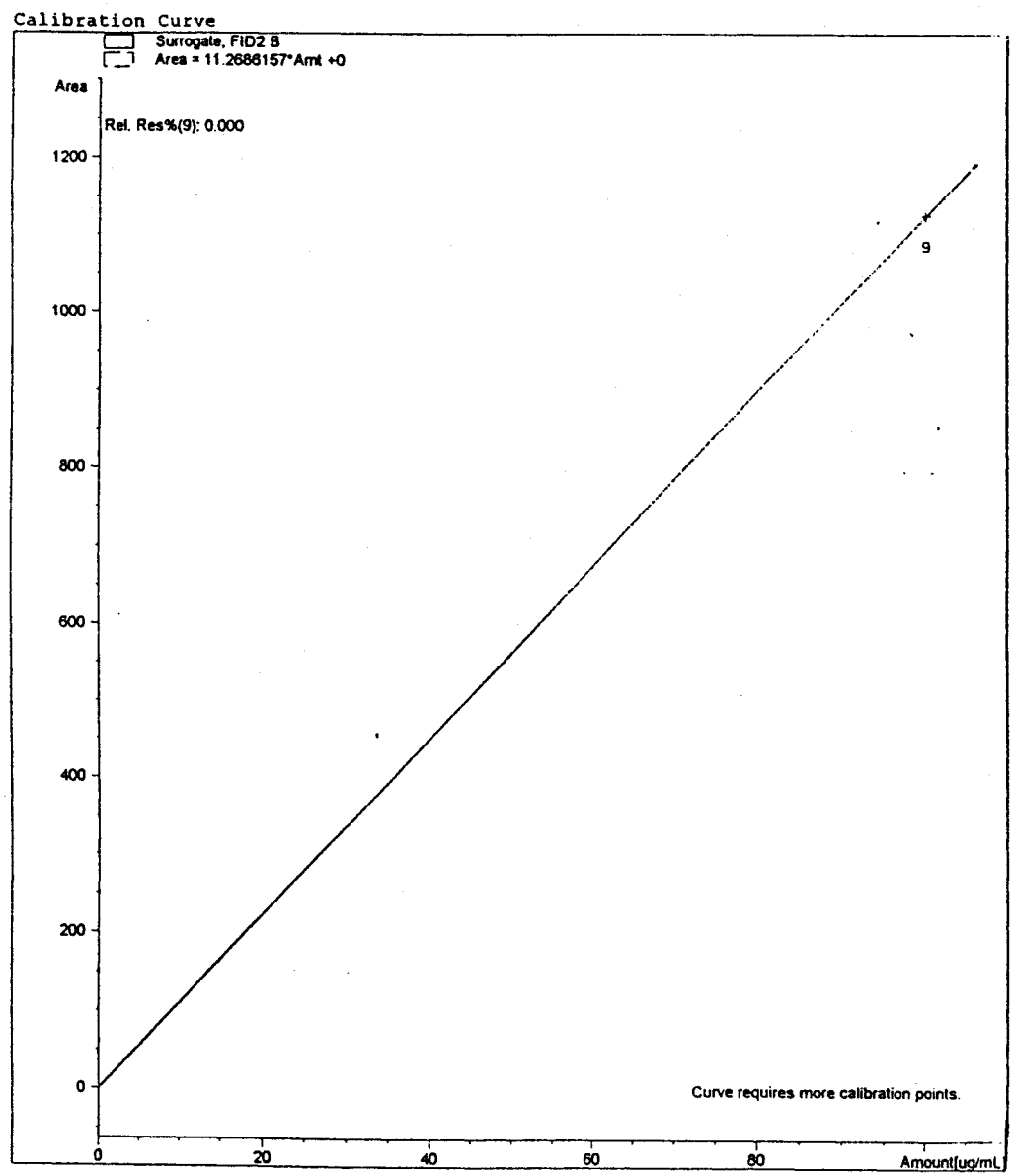
A.4

Calibration Curve of the Lubeoil range



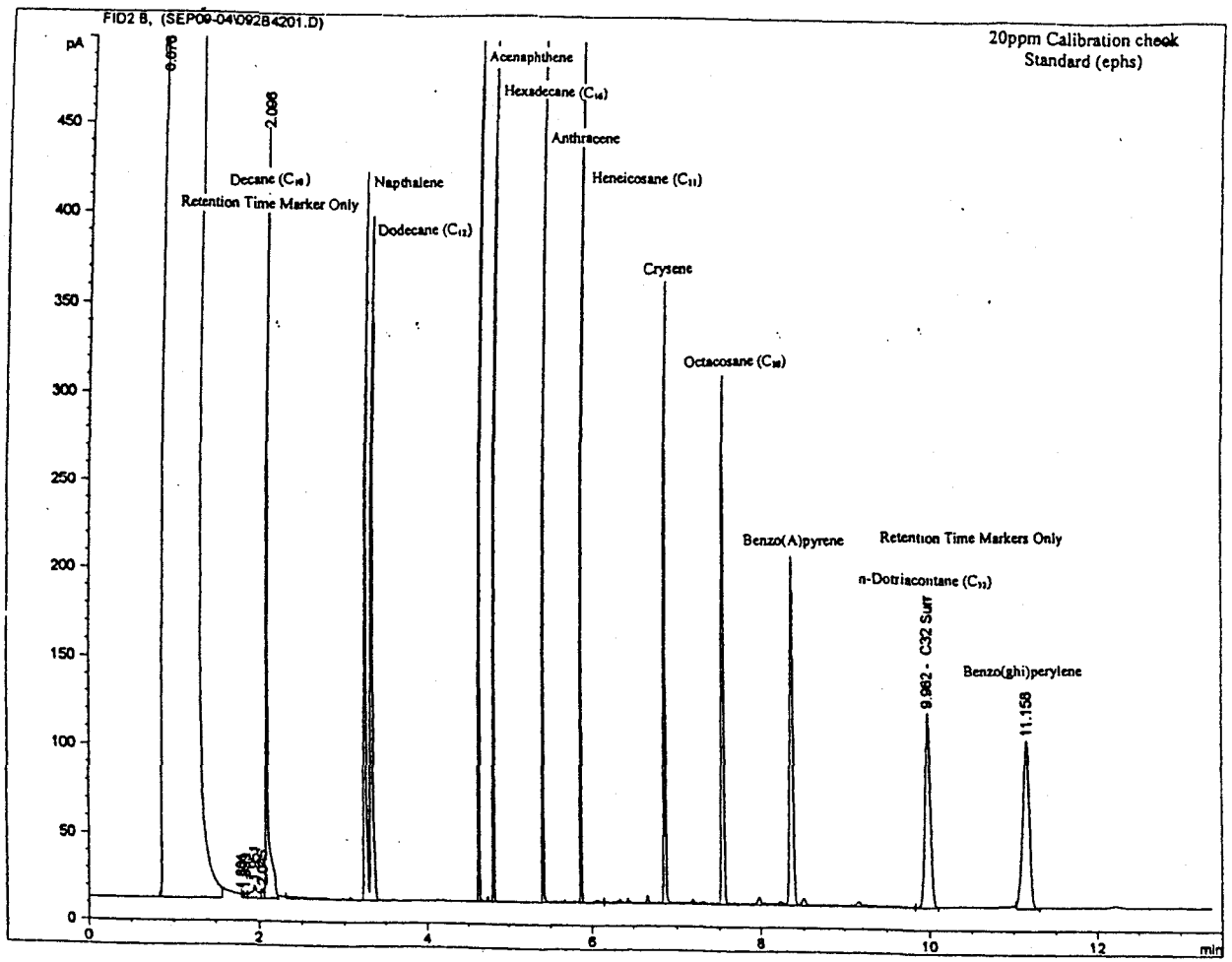
A.5

Calibration Curve of the Surrogate Compound; O-Terphenyl



A.6

Gas chromatogram
of the Fueloil range
and the Lubeoil range

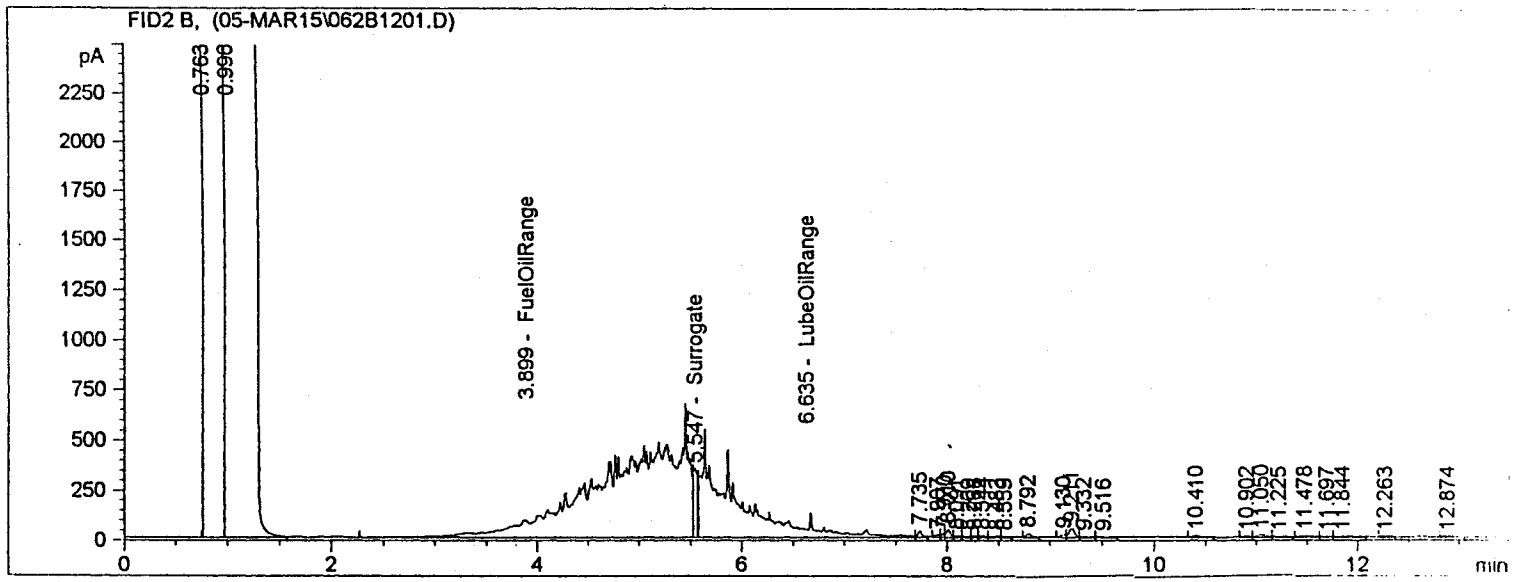


Appendix B

Phase I Biodegradation Experiments

B.1

Gas chromatogram of treatment system 1 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 2:10:41 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.899	HHA+	2.91100e4	3.41344e-2	993.65305		FuelOilRange
5.547	HH	886.74097	6.32391e-2	56.07671		Surrogate
6.635	HHA+	1.10705e4	3.69335e-2	408.87228		LubeOilRange

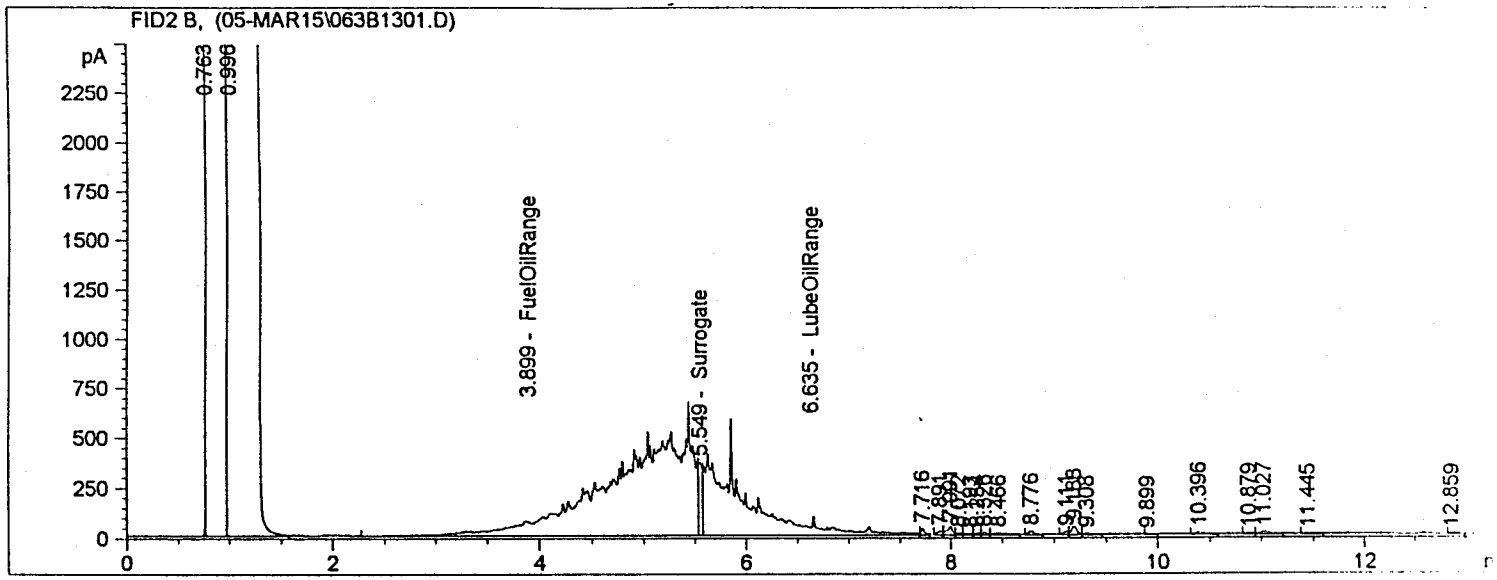
Totals : 1458.60204

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.2

Gas chromatogram of treatment system 2 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 2:10:41 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.899	HHA+	2.88379e4	3.41342e-2	984.35799		FuelOilRange
5.549	HH	922.71051	6.32391e-2	58.35139		Surrogate
6.635	HHA+	1.15039e4	3.69252e-2	424.78149		LubeOilRange

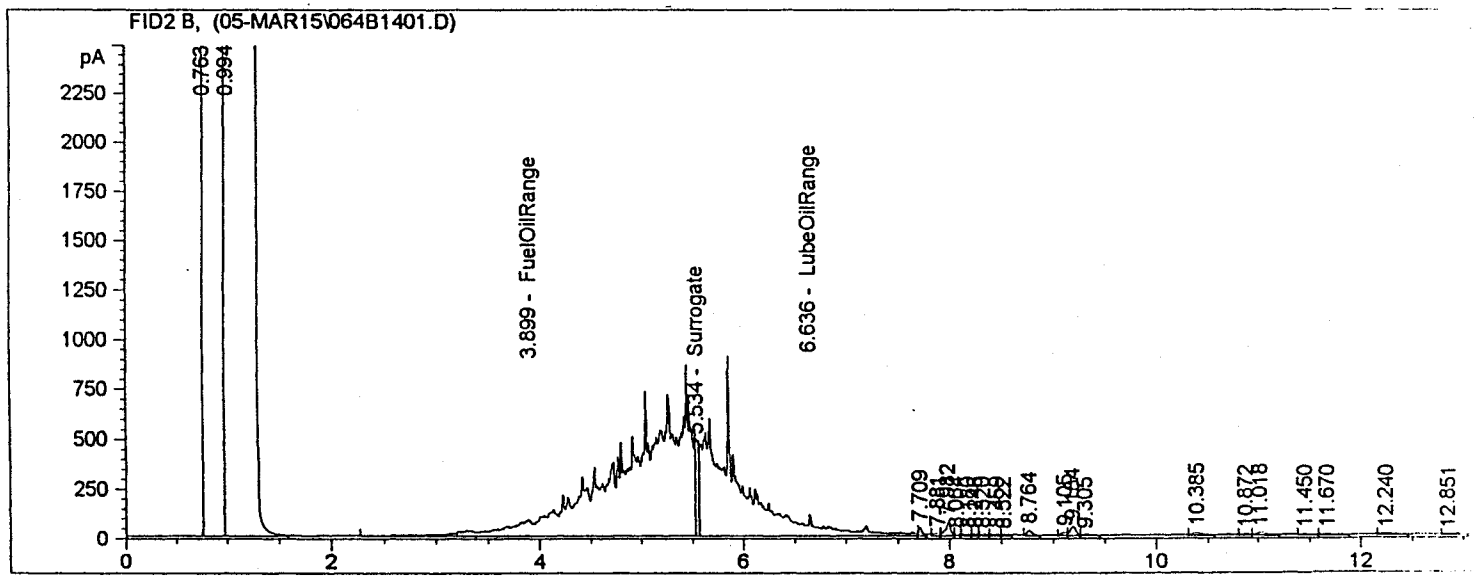
Totals : 1467.49087

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.3

Gas chromatogram of treatment system 3 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 2:10:41 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.899	HHA+	3.30642e4	3.41368e-2	1128.70458		FuelOilRange
5.534	HH	1029.34961	6.32391e-2	65.09515		Surrogate
6.636	HHA+	1.63911e4	3.68612e-2	604.19618		LubeOilRange

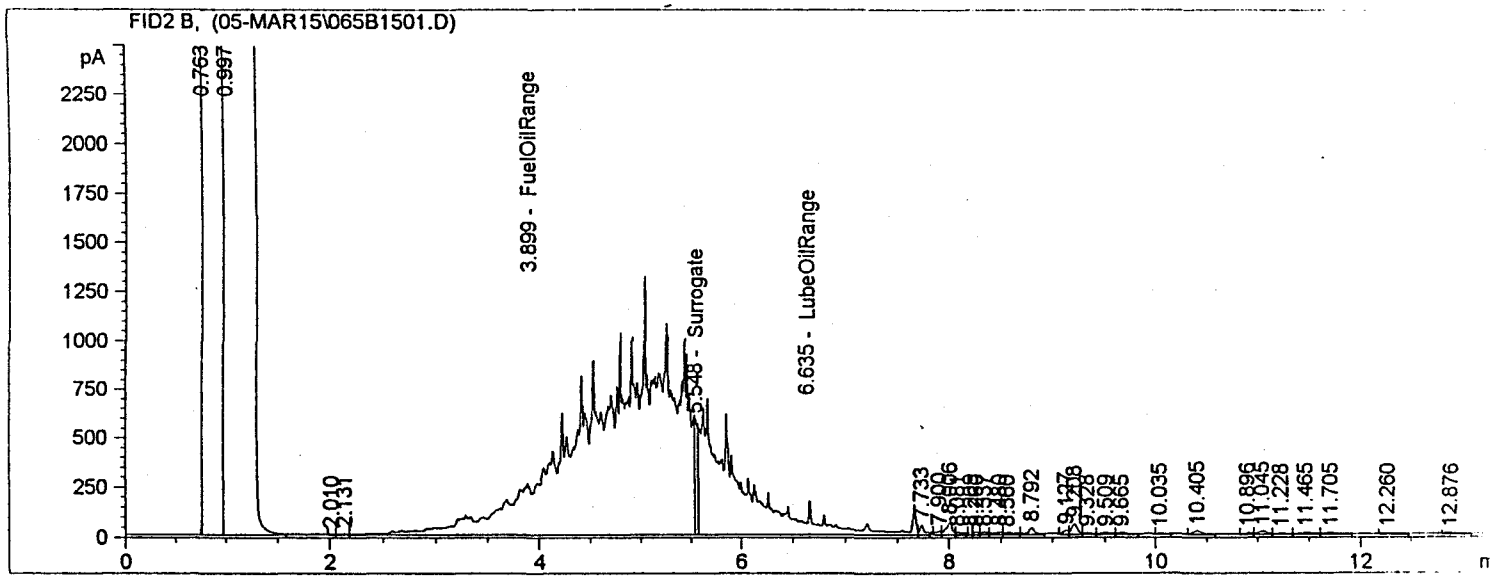
Totals : 1797.99590

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.4

Gas chromatogram of treatment system 4 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 2:10:41 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.899	HHA+	6.52924e4	3.41456e-2	2229.44930		FuelOilRange
5.548	HH	1247.50391	6.32391e-2	78.89103		Surrogate
6.635	HHA+	1.75484e4	3.68513e-2	646.68056		LubeOilRange

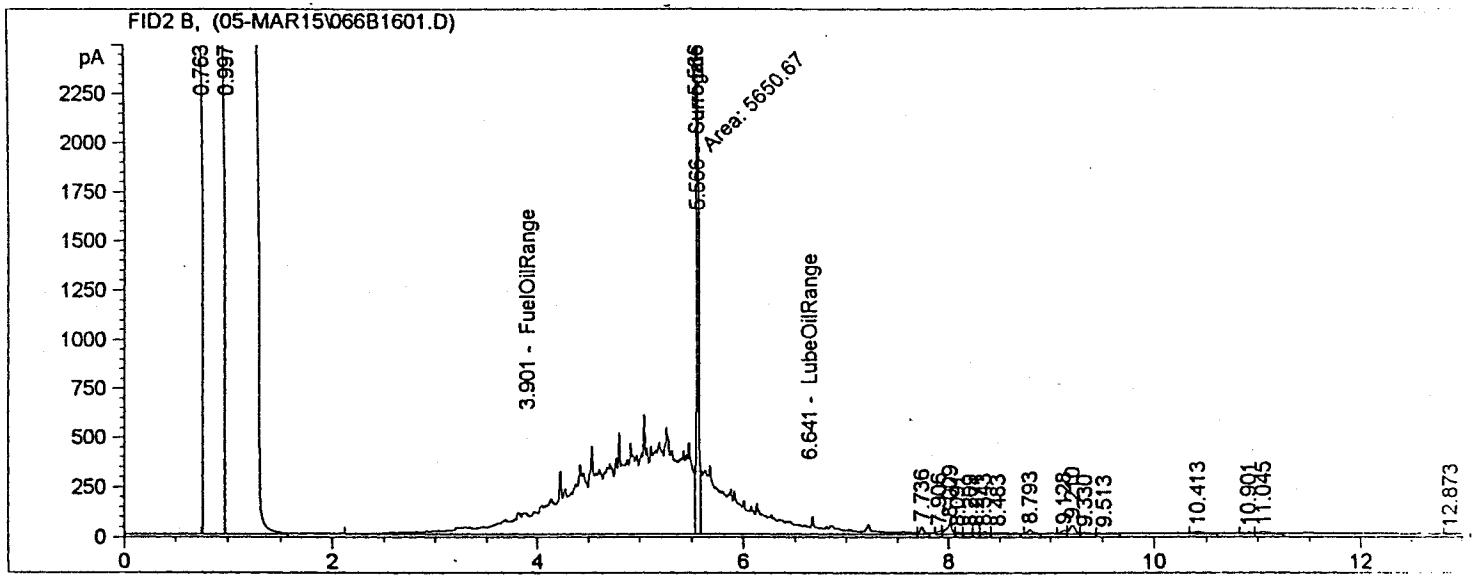
Totals : 2955.02090

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.5

Gas chromatogram of treatment system 5 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.901	HHA+	3.27836e4	3.41366e-2	1119.12367		FuelOilRange
5.566	HH S	1098.44092	6.32391e-2	69.46442		Surrogate
6.641	HHA+	9787.86035	3.69627e-2	361.78611		LubeOilRange

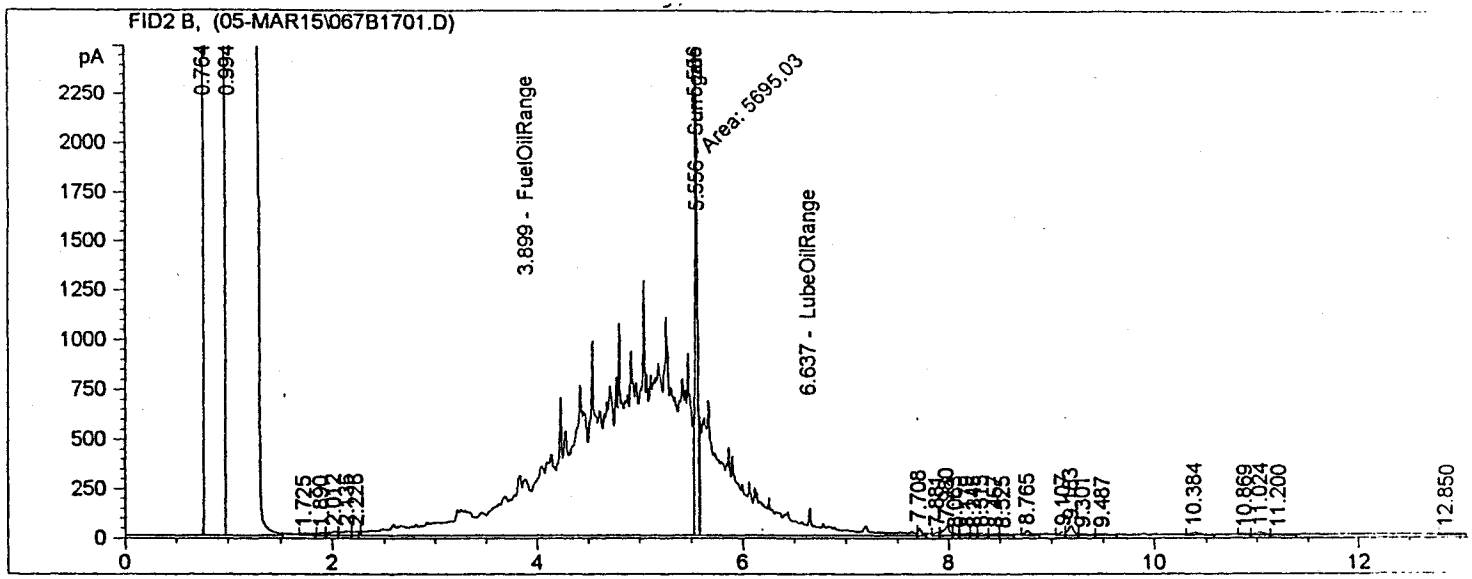
Totals : 1550.37420

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.6

Gas chromatogram of treatment system 6 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.899	HHA+	6.85548e4	3.41460e-2	2340.87330		FuelOilRange
5.556	HH S	1806.87830	6.32391e-2	114.26537		Surrogate
6.637	HHA+	1.64792e4	3.68604e-2	607.42887		LubeOilRange

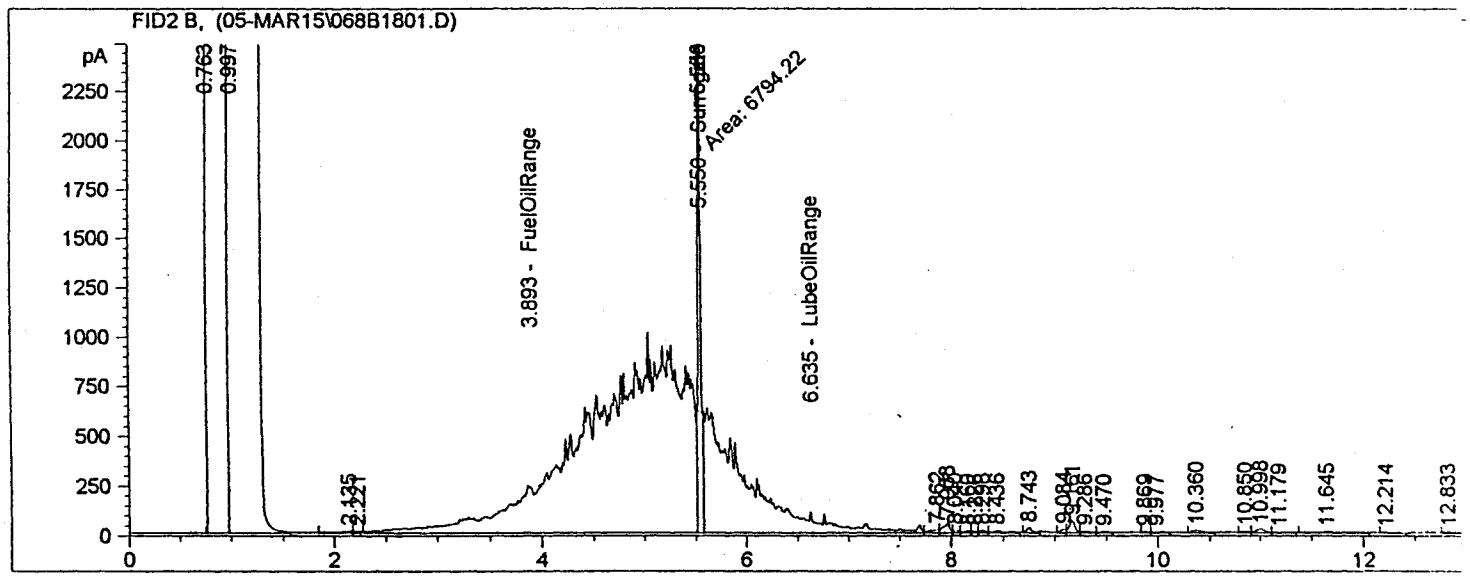
Totals : 3062.56754

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.7

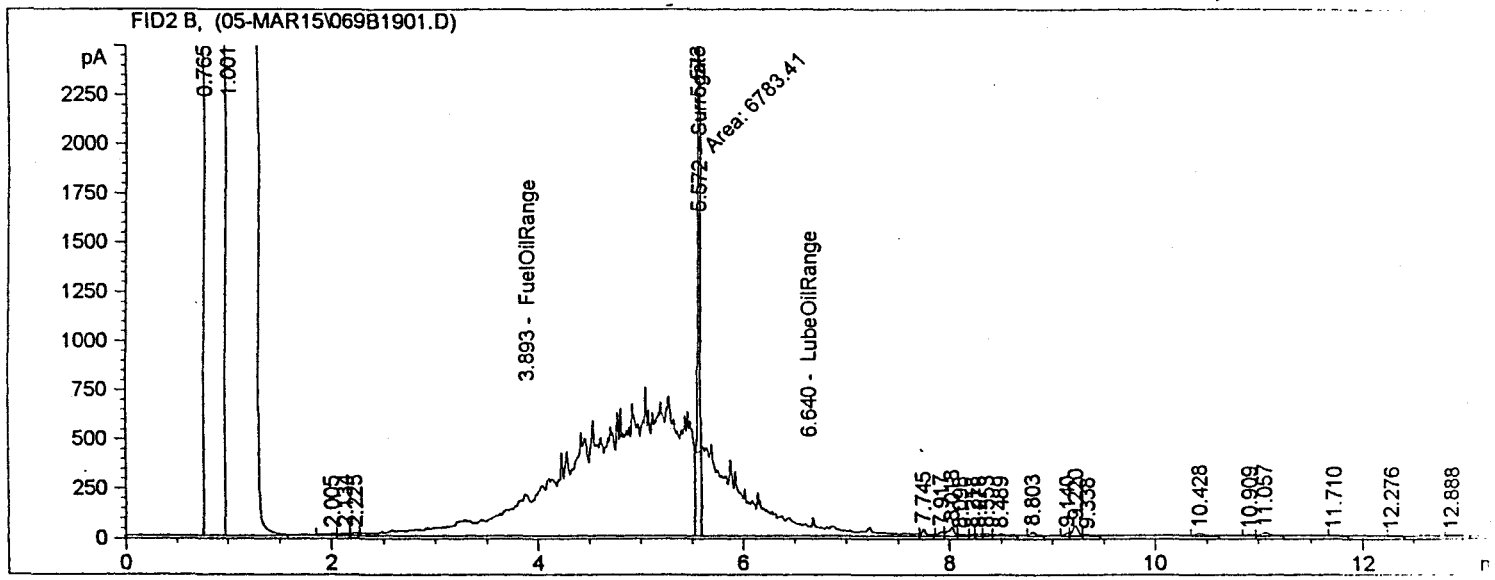
Gas chromatogram of treatment system 7 obtained in phase I experiments after 45 days




```
================================================================================
                          External Standard Report
================================================================================

Sorted By             :      Signal
Calib. Data Modified  :      05/03/16 3:48:57 PM
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID2 B,

RetTime  Type     Area      Amt/Area    Amount    Grp   Name
 [min]          [pA*s]                 [ug/mL]
-------|------|----------|----------|----------|--|------------------
  3.893 HHA+   6.43846e4  3.41455e-2 2198.44224     FuelOilRange
  5.550 HH S   2212.77124 6.32391e-2  139.93368     Surrogate
  6.635 HHA+   1.79090e4  3.68485e-2  659.92024     LubeOilRange

Totals :                              2998.29615

 Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
```

B.8

Gas chromatogram of treatment system 8 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	5.06856e4	3.41430e-2	1730.55670		FuelOilRange
5.572	HH S	1631.71912	6.32391e-2	103.18846		Surrogate
6.640	HHA+	1.38433e4	3.68889e-2	510.66258		LubeOilRange

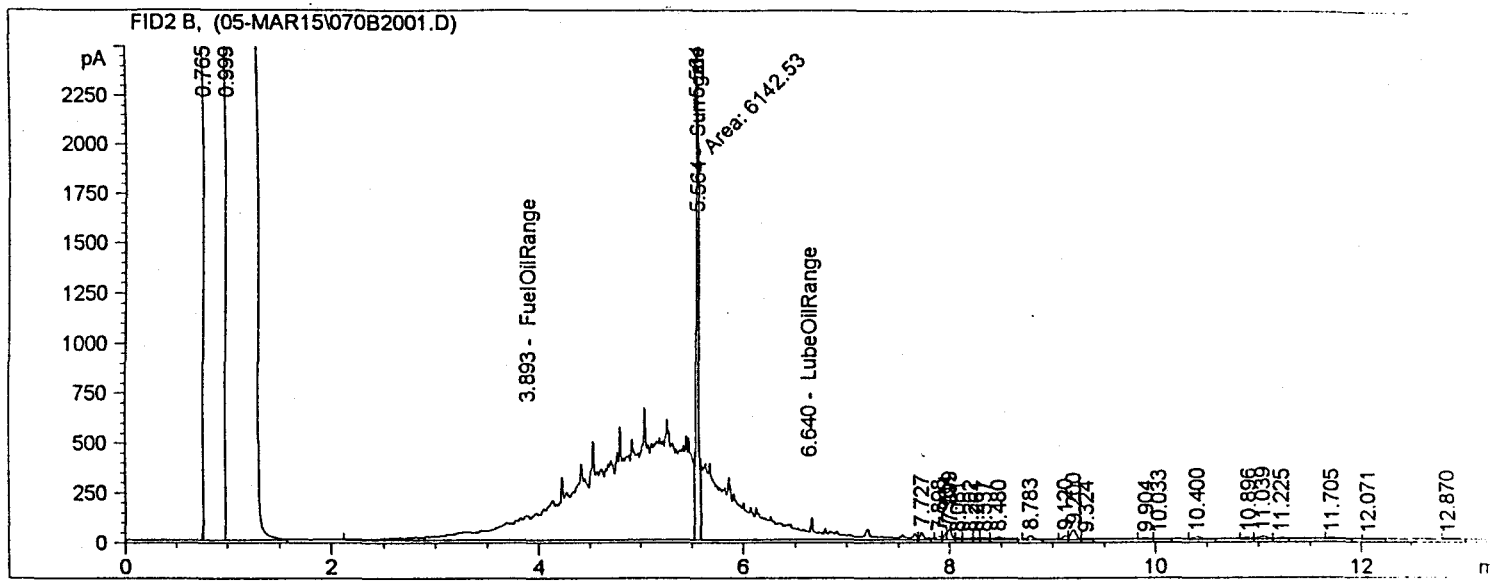
Totals : 2344.40773

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.9

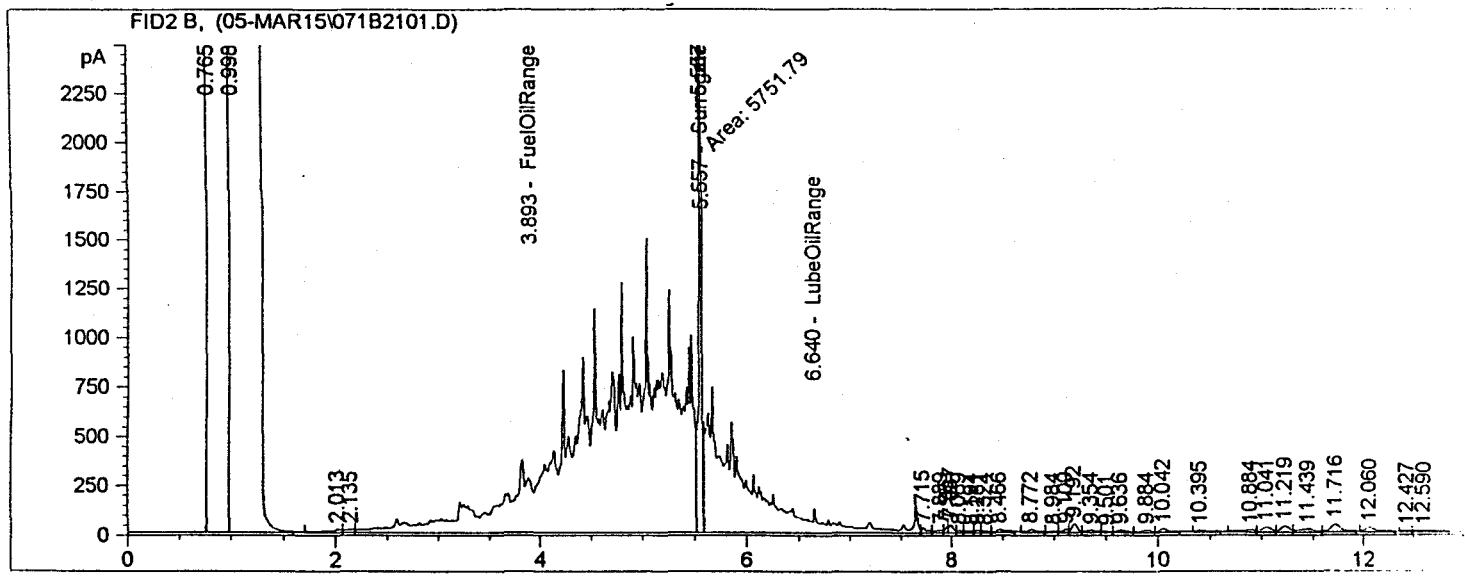
Gas chromatogram of treatment system 9 obtained in phase I experiments after 45 days




```
================================================================================
                          External Standard Report
================================================================================

Sorted By             :      Signal
Calib. Data Modified  :      05/03/16 3:48:57 PM
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID2 B,

RetTime  Type     Area      Amt/Area    Amount    Grp   Name
 [min]          [pA*s]                 [ug/mL]
-------|------|----------|----------|----------|--|------------------
  3.893 HHA+   3.80551e4  3.41391e-2 1299.16711     FuelOilRange
  5.564 HH S   1322.70850 6.32391e-2   83.64690     Surrogate
  6.640 HHA+   1.16726e4  3.69221e-2  430.97546     LubeOilRange

Totals :                              1813.78947

 Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
```

B.10

Gas chromatogram of treatment system 10 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	6.71002e4	3.41458e-2	2291.19219		FuelOilRange
5.557	HH S	2135.19995	6.32391e-2	135.02814		Surrogate
6.640	HHA+	1.69576e4	3.68562e-2	624.99325		LubeOilRange

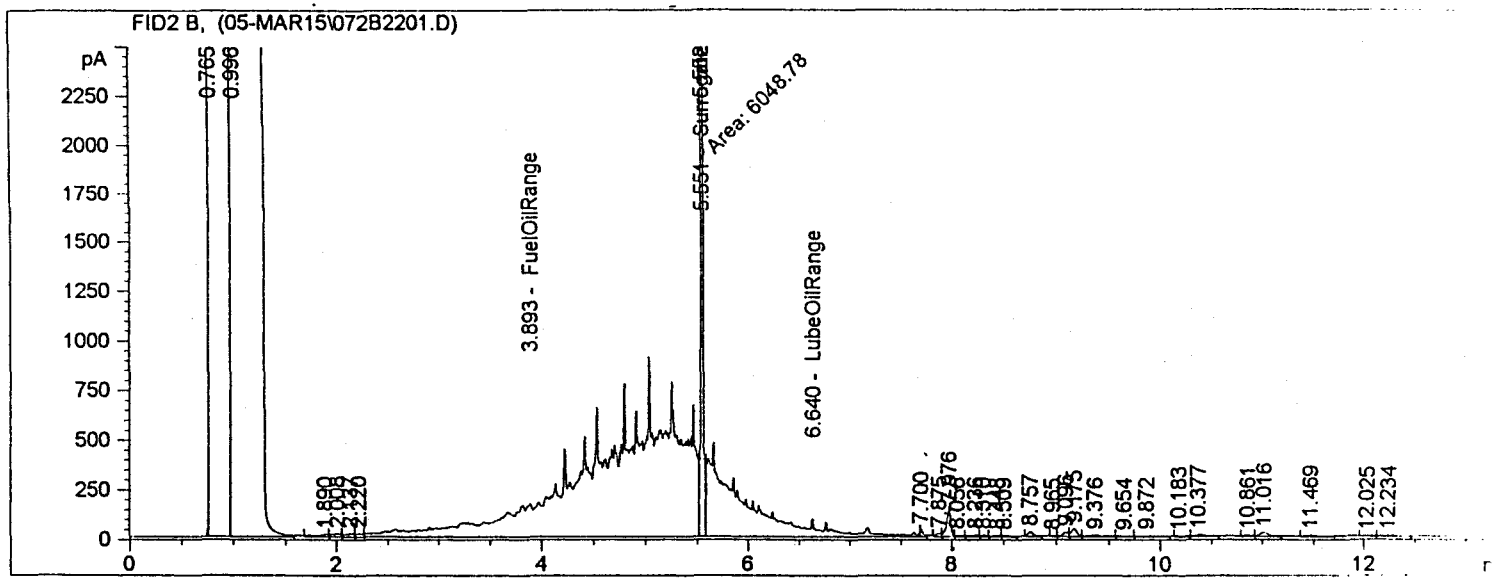
Totals : 3051.21358

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.11

Gas chromatogram of treatment system 11 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	4.36627e4	3.41411e-2	1490.69384		FuelOilRange
5.551	HH S	1387.69324	6.32391e-2	87.75648		Surrogate
6.640	HHA+	1.10733e4	3.69335e-2	408.97650		LubeOilRange

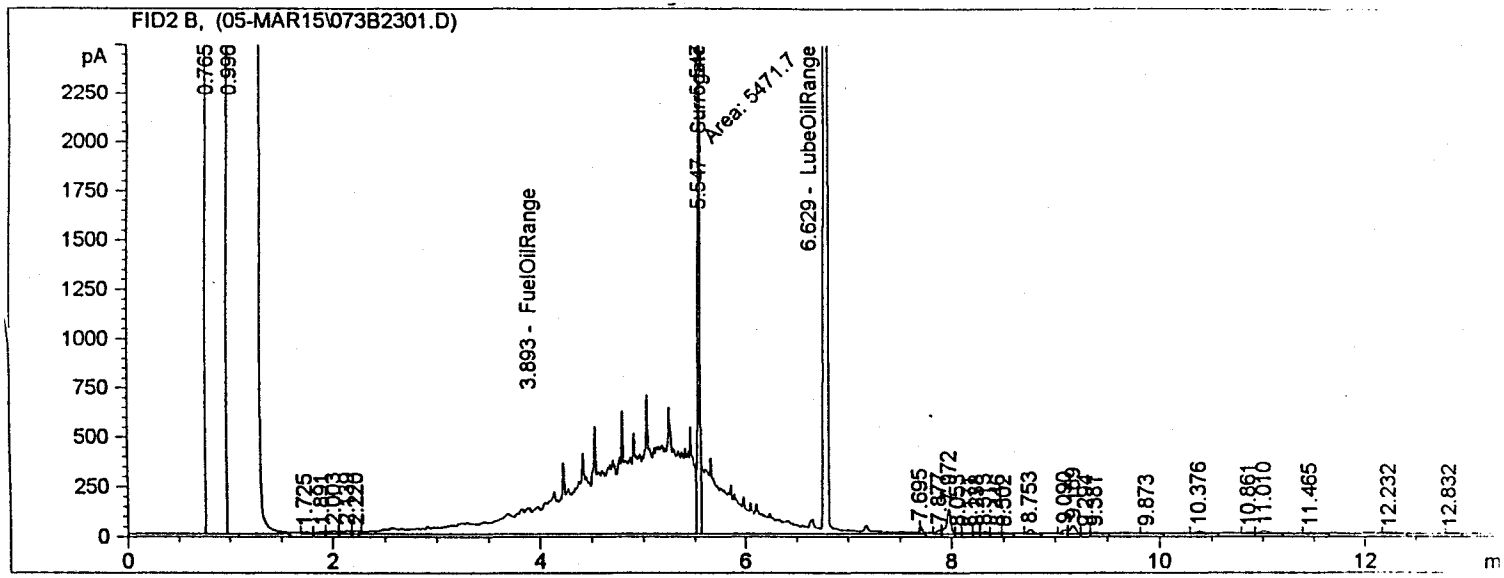
Totals : 1987.42682

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.12

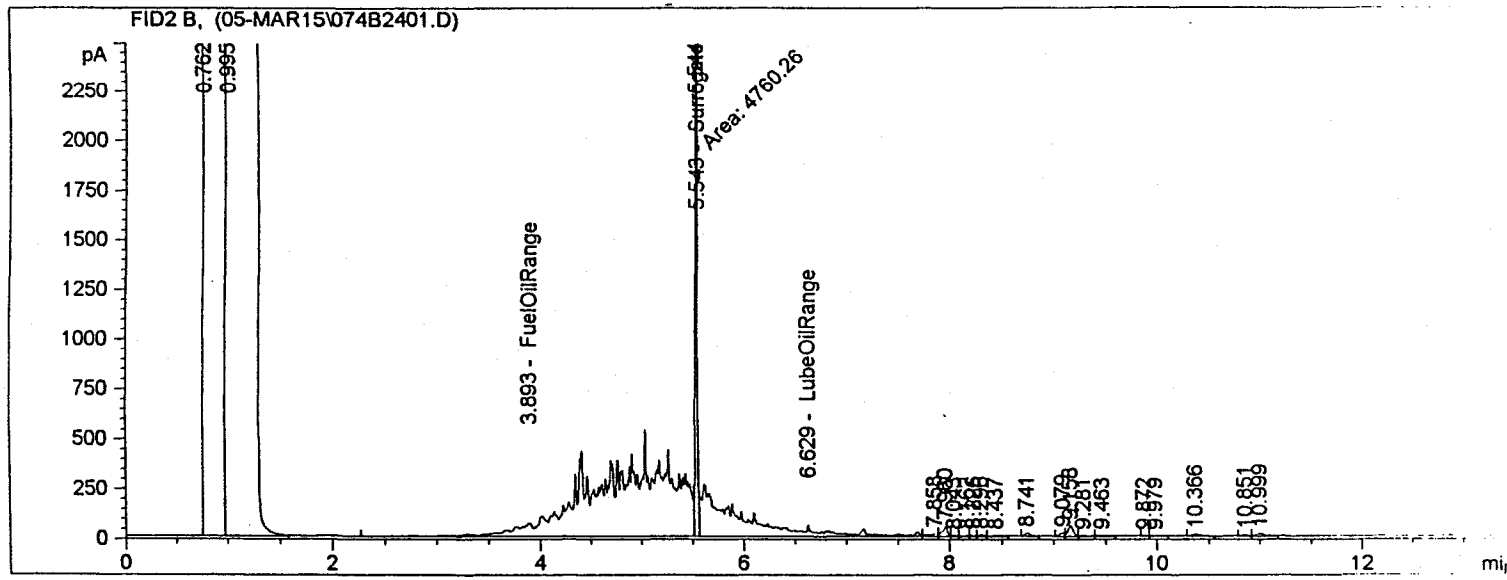
Gas chromatogram of treatment system 12 obtained in phase I experiments after 45 days




```
================================================================================
                          External Standard Report
================================================================================

Sorted By             :      Signal
Calib. Data Modified  :      05/03/16 3:48:57 PM
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID2 B,

RetTime  Type     Area      Amt/Area    Amount    Grp   Name
 [min]          [pA*s]                 [ug/mL]
-------|------|----------|----------|----------|--|------------------
  3.893 HHA+   3.58676e4  3.41382e-2 1224.45627    FuelOilRange
  5.547 HH S    985.03137 6.32391e-2   62.29250    Surrogate
  6.629 HHA+   2.71912e4  3.68015e-2 1000.67574    LubeOilRange

Totals :                               2287.42451

 Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
```

B.13

Gas chromatogram of treatment system 13 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	2.31796e4	3.41292e-2	791.10092		FuelOilRange
5.543	HH S	601.80682	6.32391e-2	38.05773		Surrogate
6.629	HHA+	6538.09766	3.70880e-2	242.48486		LubeOilRange

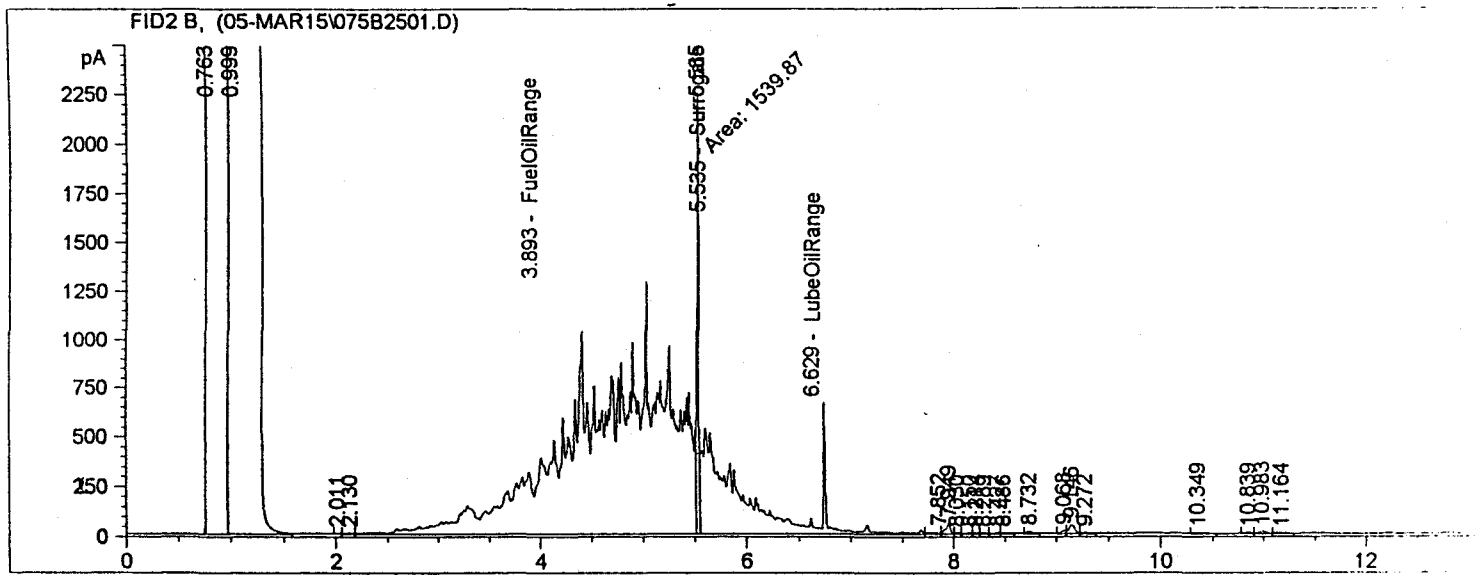
Totals : 1071.64350

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.14

Gas chromatogram of treatment system 14 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	6.15655e4	3.41450e-2	2102.15702		FuelOilRange
5.535	MM	1539.86914	6.32391e-2	97.37995		Surrogate
6.629	HHA+	1.29630e4	3.69010e-2	478.34872		LubeOilRange

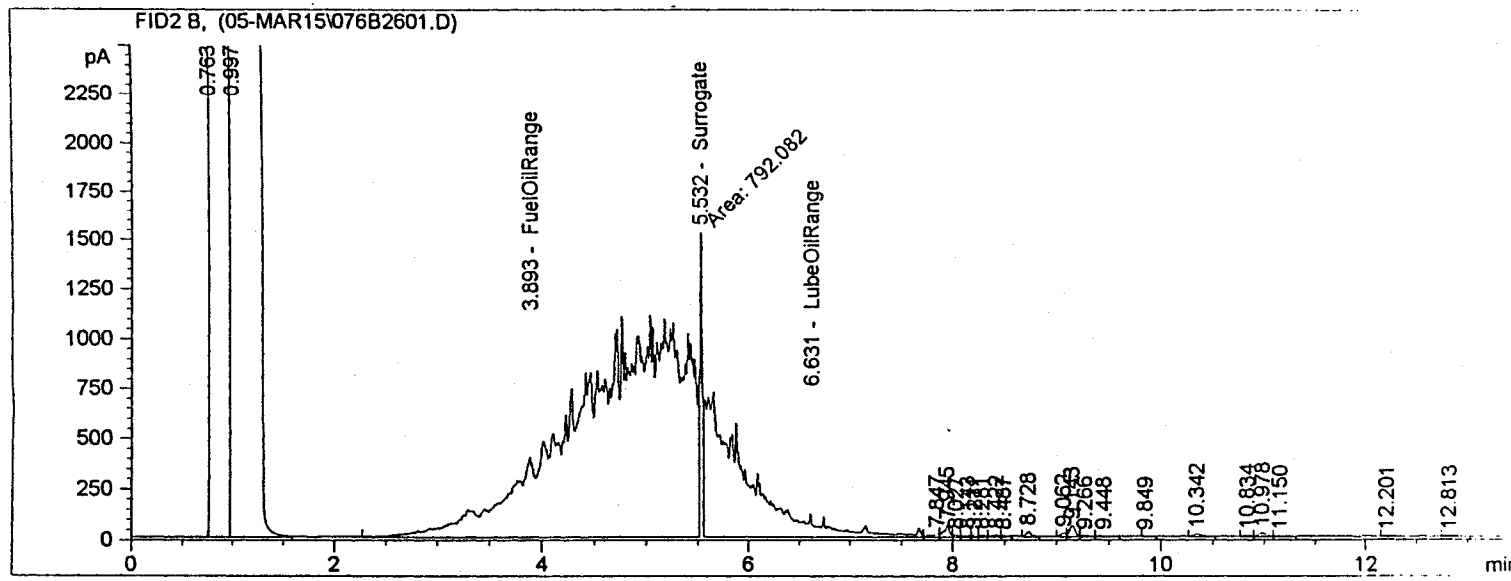
Totals : 2677.88569

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.15

Gas chromatogram of treatment system 15 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	8.04147e4	3.41473e-2	2745.94555		FuelOilRange
5.532	MM	792.08179	6.32391e-2	50.09054		Surrogate
6.631	HHA+	1.98812e4	3.68348e-2	732.31866		LubeOilRange

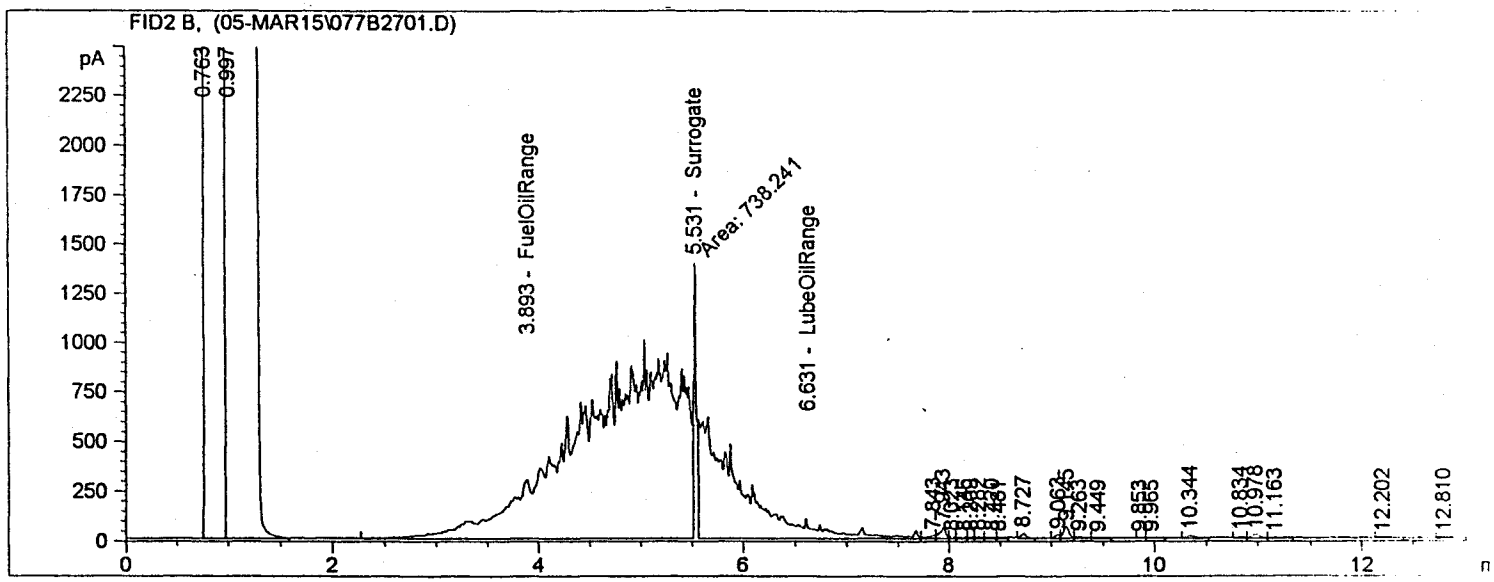
Totals : 3528.35475

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.16

Gas chromatogram of treatment system 16 obtained in phase I experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	6.60407e4	3.41457e-2	2255.00723		FuelOilRange
5.531	MM	738.24084	6.32391e-2	46.68569		Surrogate
6.631	HHA+	1.70015e4	3.68558e-2	626.60444		LubeOilRange

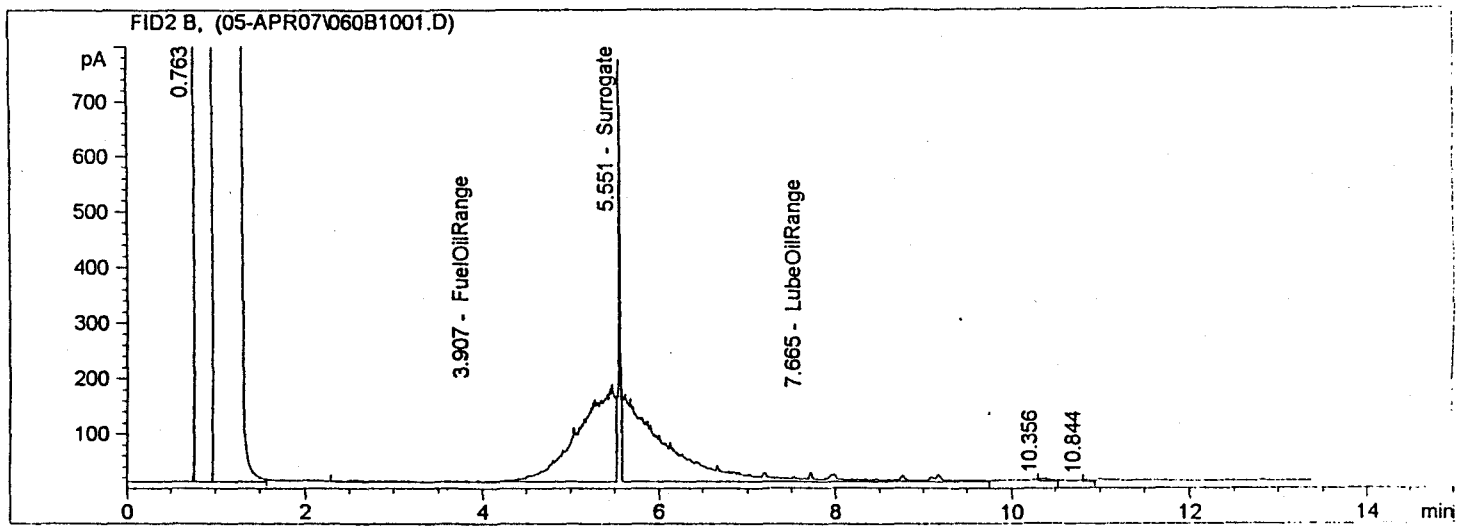
Totals : 2928.29736

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

B.17

Gas chromatogram of treatment system 17 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:16:44 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

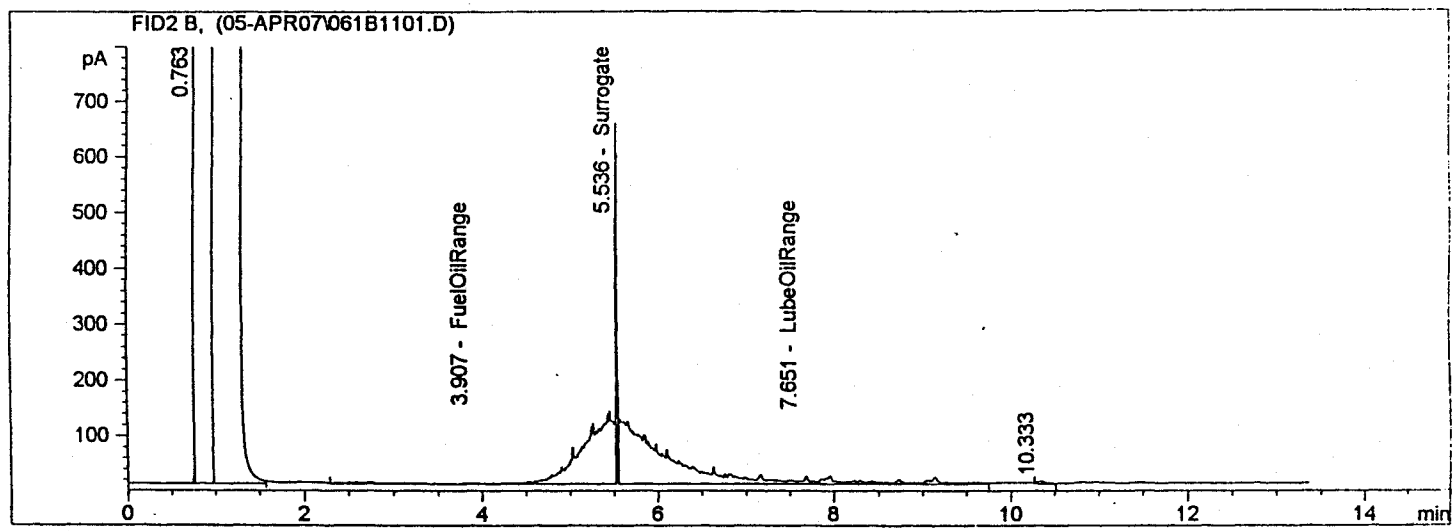
Signal Description : FID2 B,

R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
3.907	HHA+	5157.04590	175.54742	FuelOilRange
5.551	MM	471.98608	41.88501	Surrogate
7.665	HHA+	5806.71289	215.63517	LubeOilRange
Totals:			433.06760	

Results obtained with enhanced integrator!

B.18

Gas chromatogram of treatment system 18 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

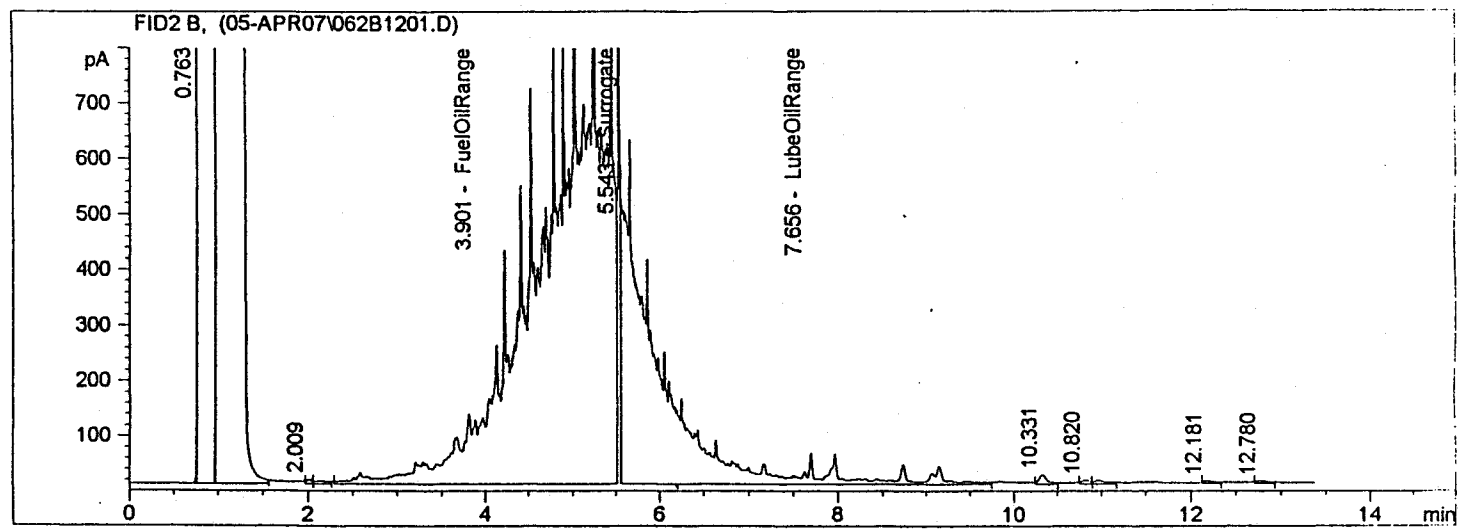
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.907	HHA+	3257.02466	110.65291	FuelOilRange
2	5.536	MM	405.07236	35.94695	Surrogate
3	7.651	HHA+	4753.20703	176.96017	LubeOilRange

Totals: 323.56003

Results obtained with enhanced integrator!

B.19

Gas chromatogram of treatment system 19 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

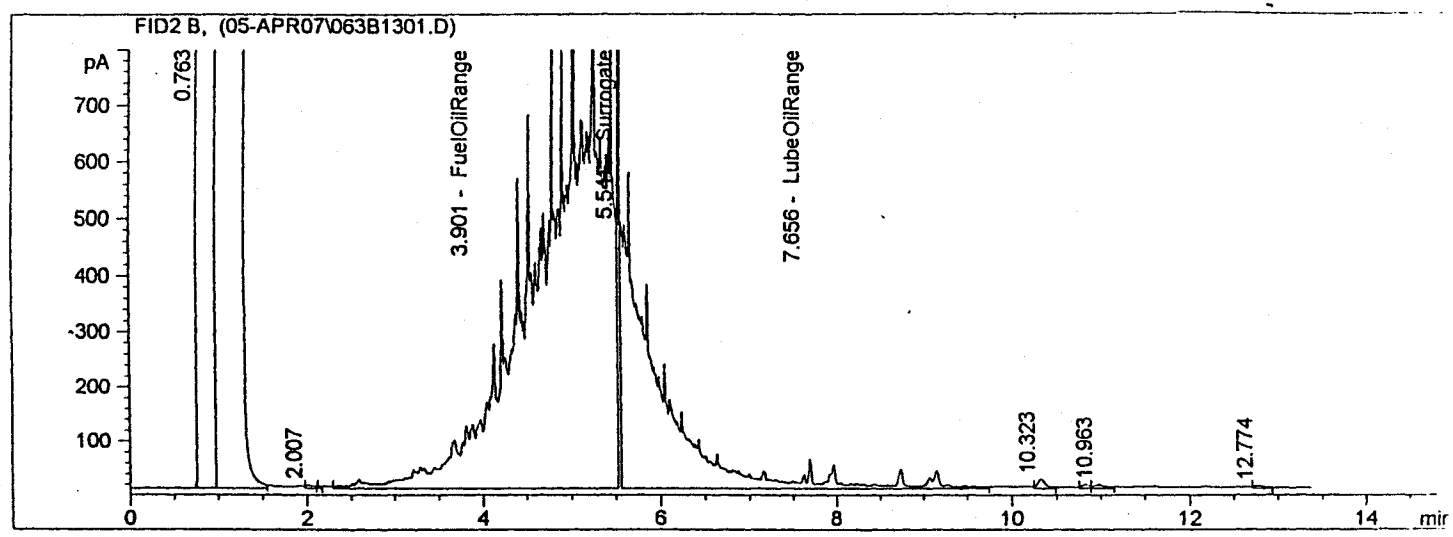
Signal Description : FID2 B,

R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
3.901	HHA+	4.49847e4	1535.84585	FuelOilRange
5.543	MM	431.94568	38.33174	Surrogate
7.656	HHA+	1.60194e4	590.54999	LubeOilRange
Totals:			2164.72758	

Results obtained with enhanced integrator!

B.20

Gas chromatogram of treatment system 20 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

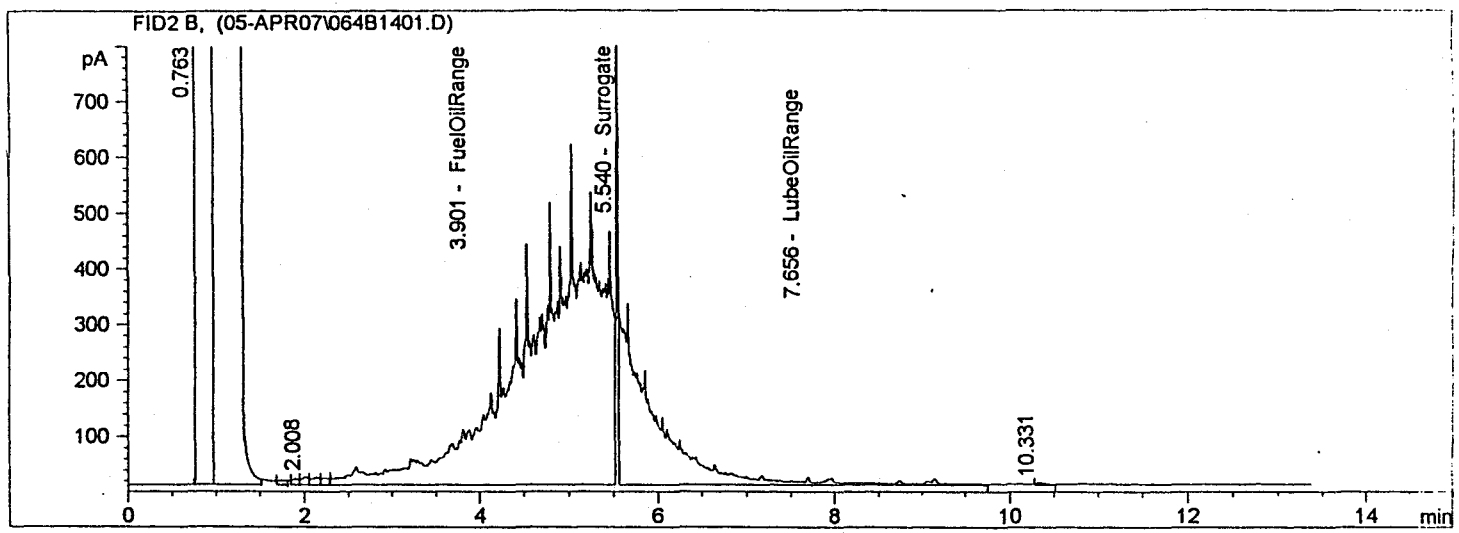
```
Sorted By             :  Signal
Calib. Data Modified  :  Thu, Apr. 14, 2005 8:26:34 pm
Multiplier            :  1.000000
Dilution              :  1.000000
Uncalibrated Peaks    :  not reported

Signal Description : FID2 B,
```

	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.901	HHA+	4.43766e4	1515.07770	FuelOilRange
2	5.541	MM	1002.08203	88.92681	Surrogate
3	7.656	HHA+	1.47093e4	542.45639	LubeOilRange
	Totals:			2146.46090	

Results obtained with enhanced integrator!

B.21

Gas chromatogram of treatment system 21 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By	: Signal
Calib. Data Modified	: Thu, Apr. 14, 2005 8:26:34 pm
Multiplier	: 1.000000
Dilution	: 1.000000
Uncalibrated Peaks	: not reported

Signal Description : FID2 B,

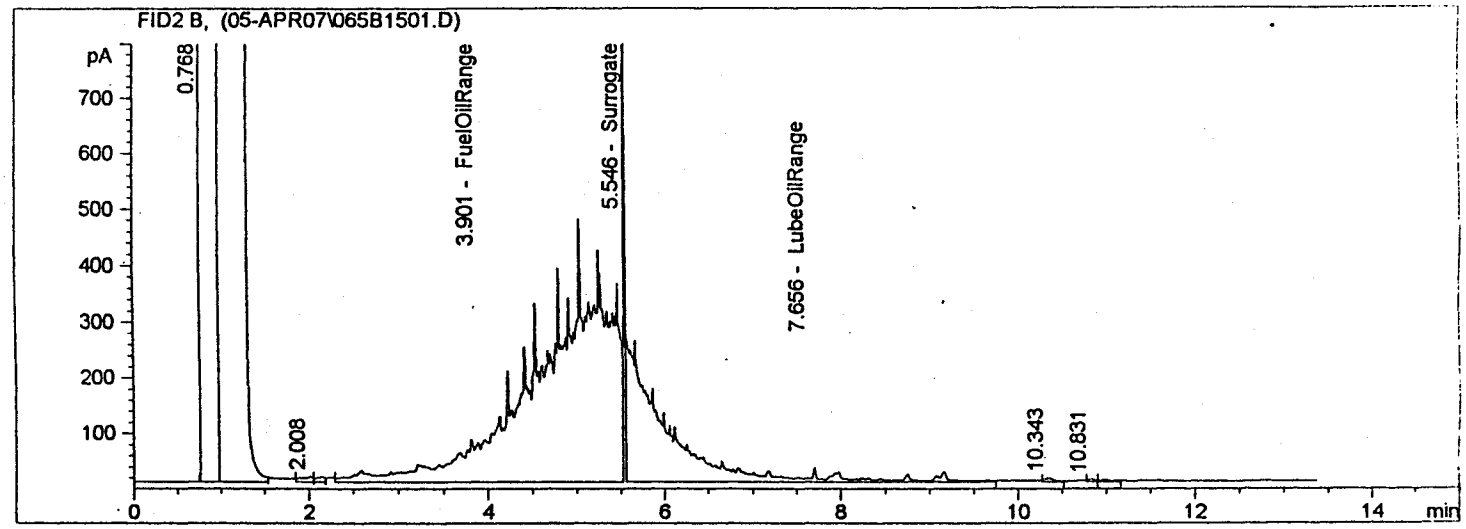
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	2.96942e4	1013.60403	FuelOilRange
.|	5.540	MM	910.82355	80.82834	Surrogate
.|	7.656	HHA+	8581.07031	317.48393	LubeOilRange

Totals:	1411.91630

Results obtained with enhanced integrator!

B.22

Gas chromatogram of treatment system 22 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

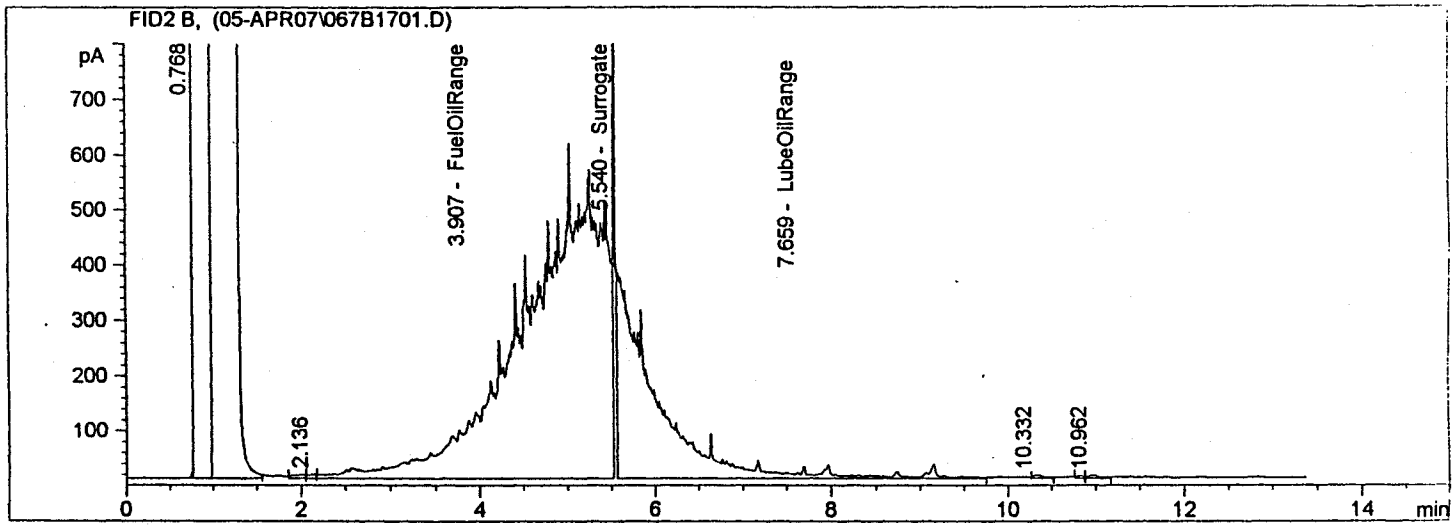
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	2.32065e4	792.01923	FuelOilRange
.|	5.546	MM	985.85486	87.48678	Surrogate
.|	7.656	HHA+	7903.90088	292.62453	LubeOilRange

Totals: 1172.13053

Results obtained with enhanced integrator!

B.23

Gas chromatogram of treatment system 23 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

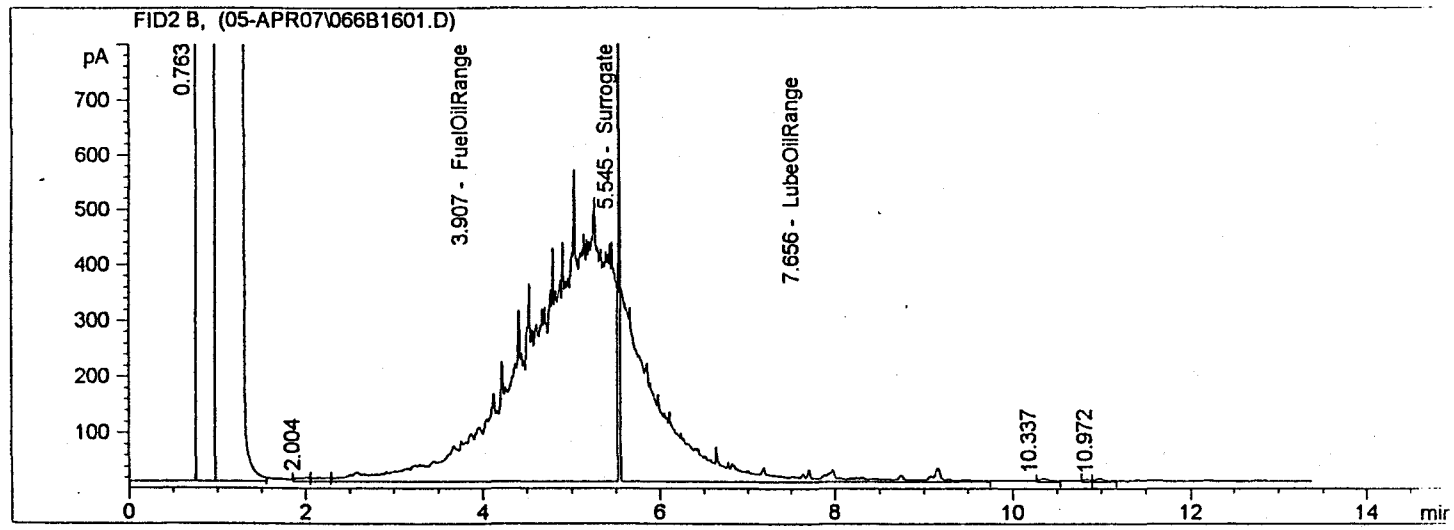
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	3.50734e4	1197.32856	FuelOilRange
.|	5.540	MM	776.03815	68.86721	Surrogate
.|	7.659	HHA+	1.13935e4	420.73072	LubeOilRange

Totals: 1686.92649

Results obtained with enhanced integrator!

B.24

Gas chromatogram of treatment system 24 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

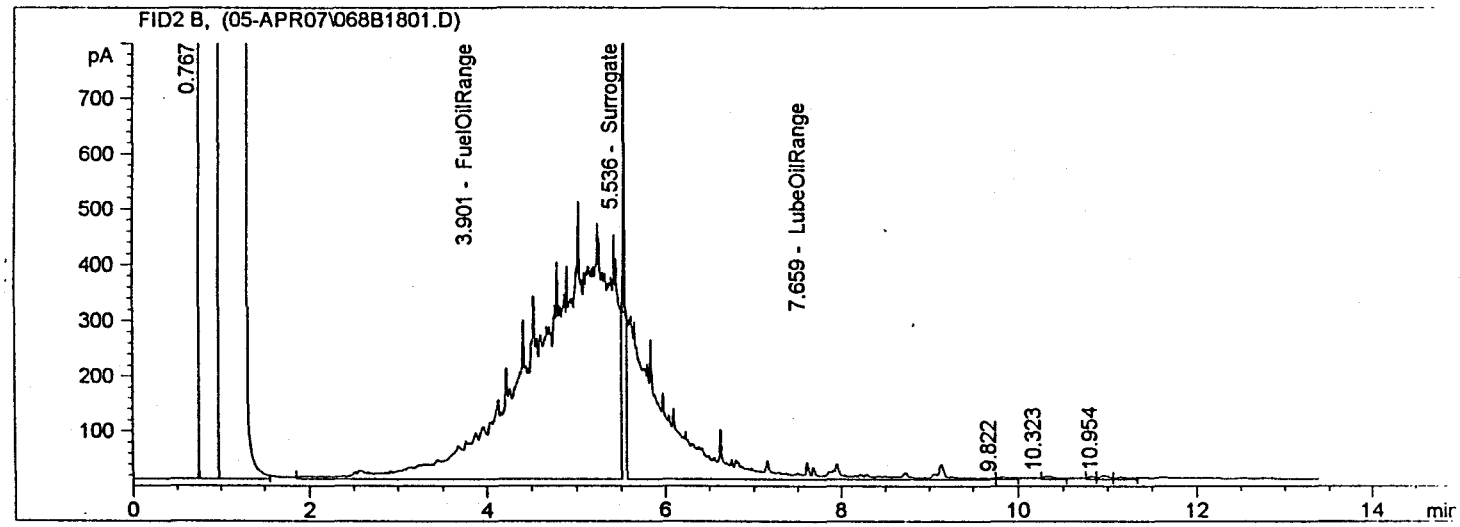
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
	3.907	HHA+	3.04203e4	1038.40288	FuelOilRange
	5.545	MM	724.22900	64.26956	Surrogate
	7.656	HHA+	1.05100e4	388.29580	LubeOilRange

Totals: 1490.96824

Results obtained with enhanced integrator!

B.25

Gas chromatogram of treatment system 25 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

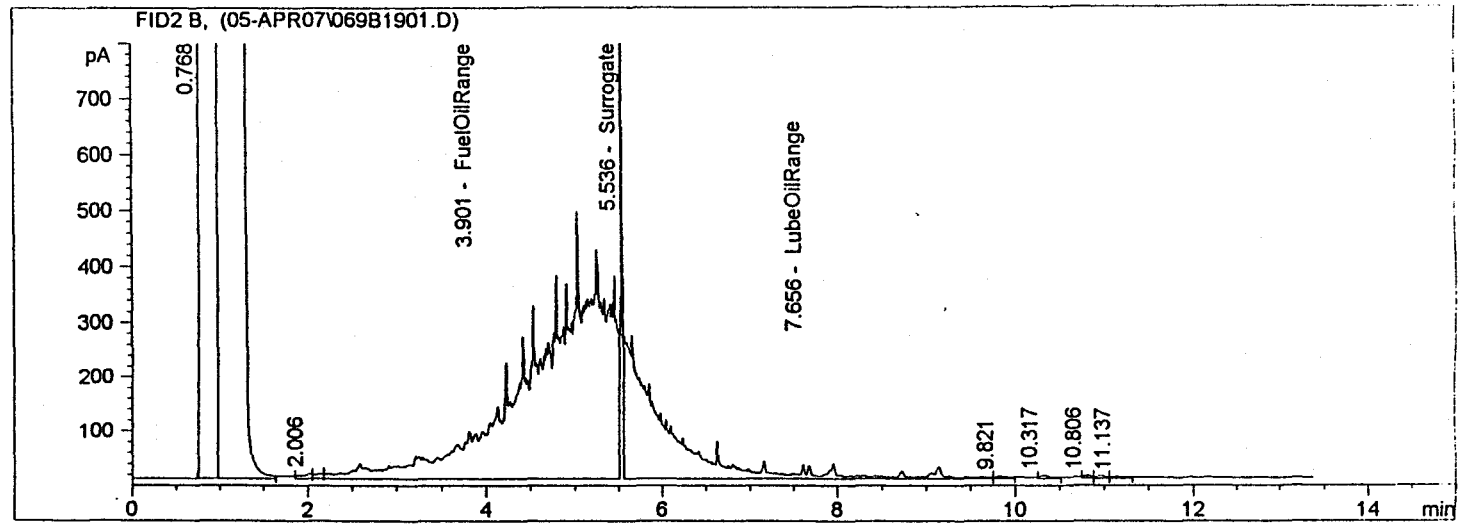
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
	3.901	HHA+	2.77462e4	947.07315	FuelOilRange
	5.536	MM	811.56500	72.01994	Surrogate
	7.659	HHA+	9658.72363	357.04540	LubeOilRange

Totals: 1376.13849

Results obtained with enhanced integrator!

B.26

Gas chromatogram of treatment system 26 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

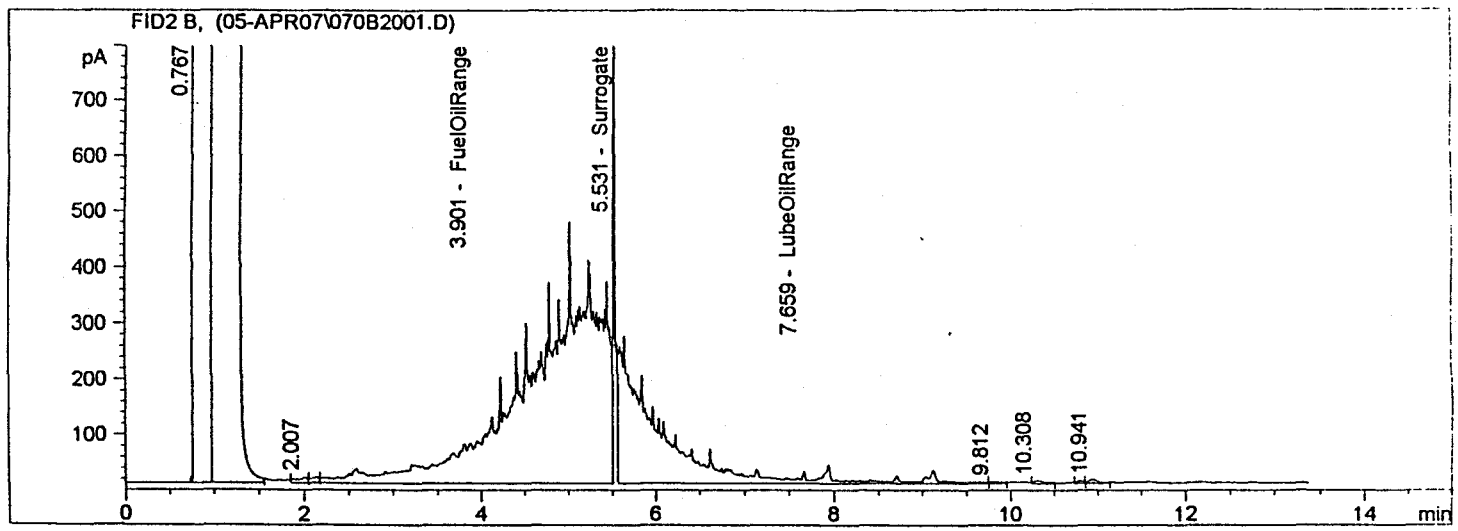
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.901	HHA+	2.50569e4	855.21922	FuelOilRange
2	5.536	MM	652.79926	57.93074	Surrogate
3	7.656	HHA+	8235.05469	304.78144	LubeOilRange

Totals: 1217.93140

Results obtained with enhanced integrator!

B.27

Gas chromatogram of treatment system 27 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

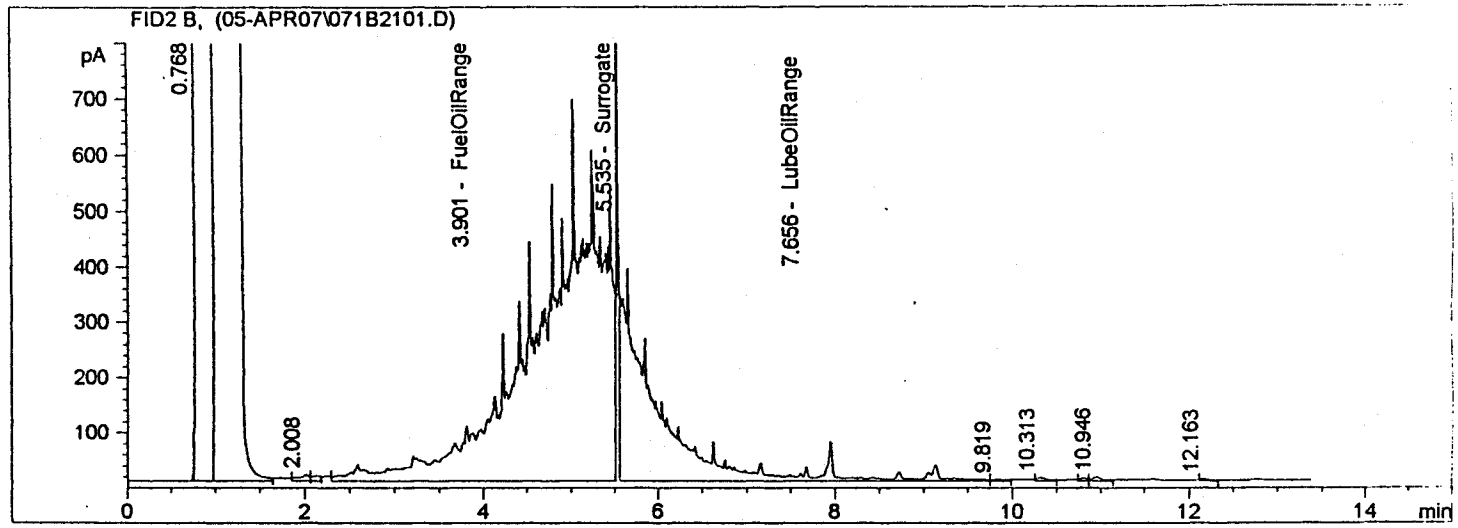
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	2.31803e4	791.12600	FuelOilRange
.|	5.531	MM	791.05298	70.19966	Surrogate
.|	7.659	HHA+	8178.03418	302.68817	LubeOilRange

Totals: 1164.01383

Results obtained with enhanced integrator!

B.28

Gas chromatogram of treatment system 28 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

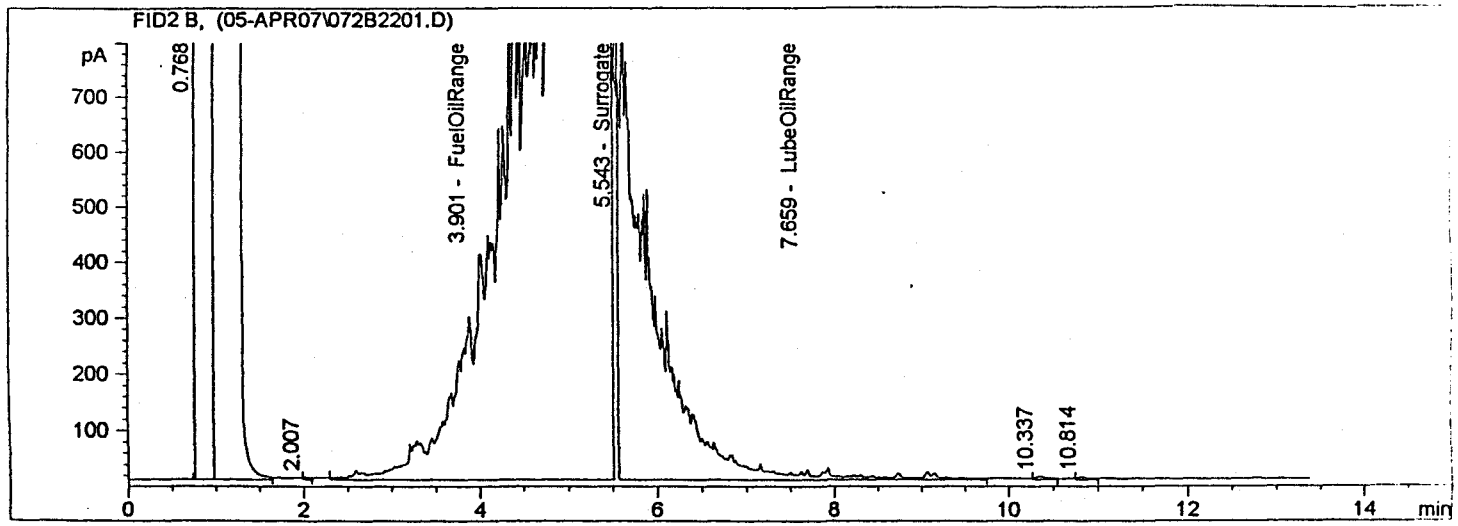
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	3.06948e4	1047.77973	FuelOilRange
.|	5.535	MM	794.88513	70.53973	Surrogate
.|	7.656	HHA+	1.02858e4	380.06728	LubeOilRange

Totals: 1498.38674

Results obtained with enhanced integrator!

B.29

Gas chromatogram of treatment system 29 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

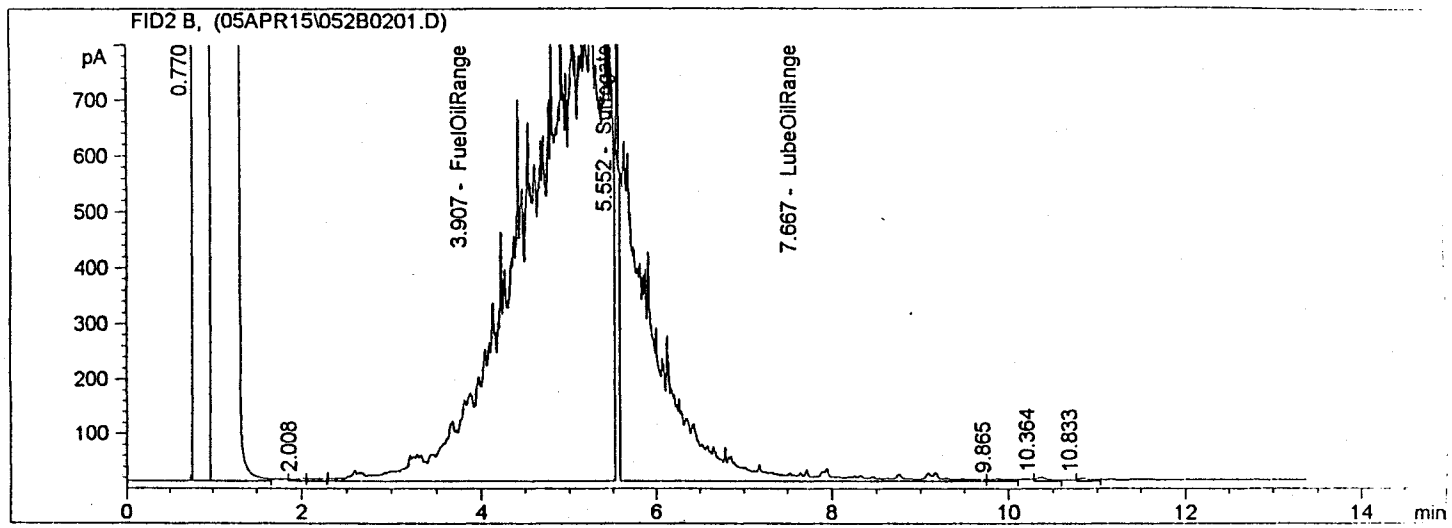
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	8.11362e4	2770.58757	FuelOilRange
.|	5.543	MM	832.83844	73.90779	Surrogate
.|	7.659	HHA+	2.03683e4	750.20131	LubeOilRange

Totals: 3594.69667

Results obtained with enhanced integrator!

B.30

Gas chromatogram of treatment system 30 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

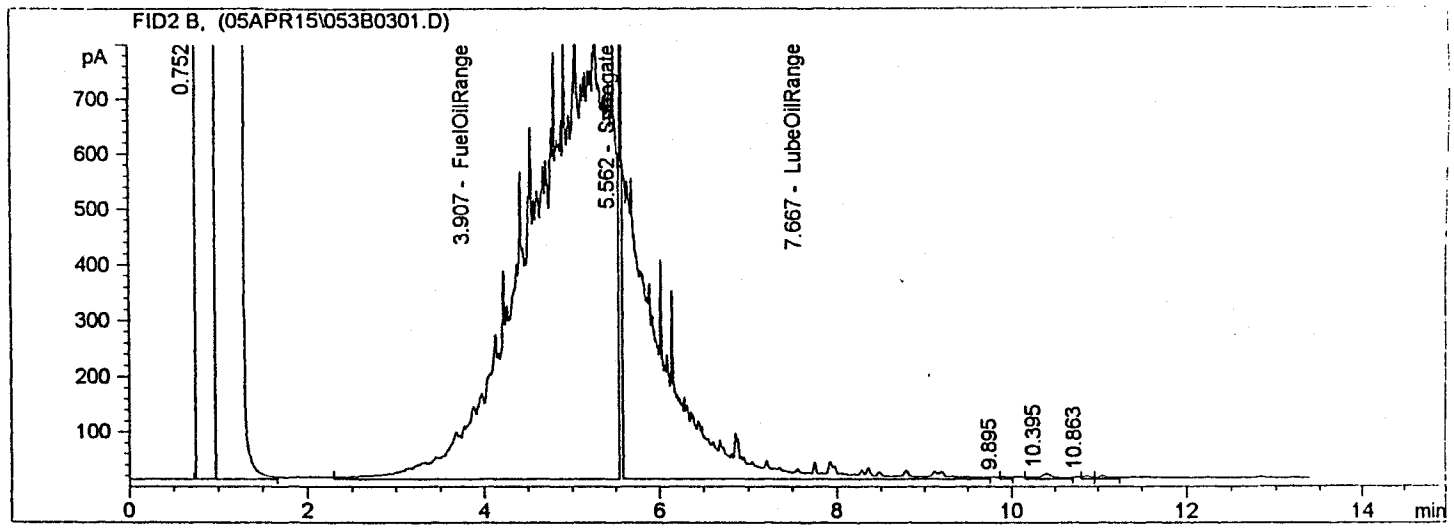
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.907	HHA+	5.68297e4	1940.40832	FuelOilRange
2	5.552	MM	800.42181	71.03107	Surrogate
3	7.667	HHA+	1.69719e4	625.51688	LubeOilRange

Totals: 2636.95627

Results obtained with enhanced integrator!

B.31

Gas chromatogram of treatment system 31 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

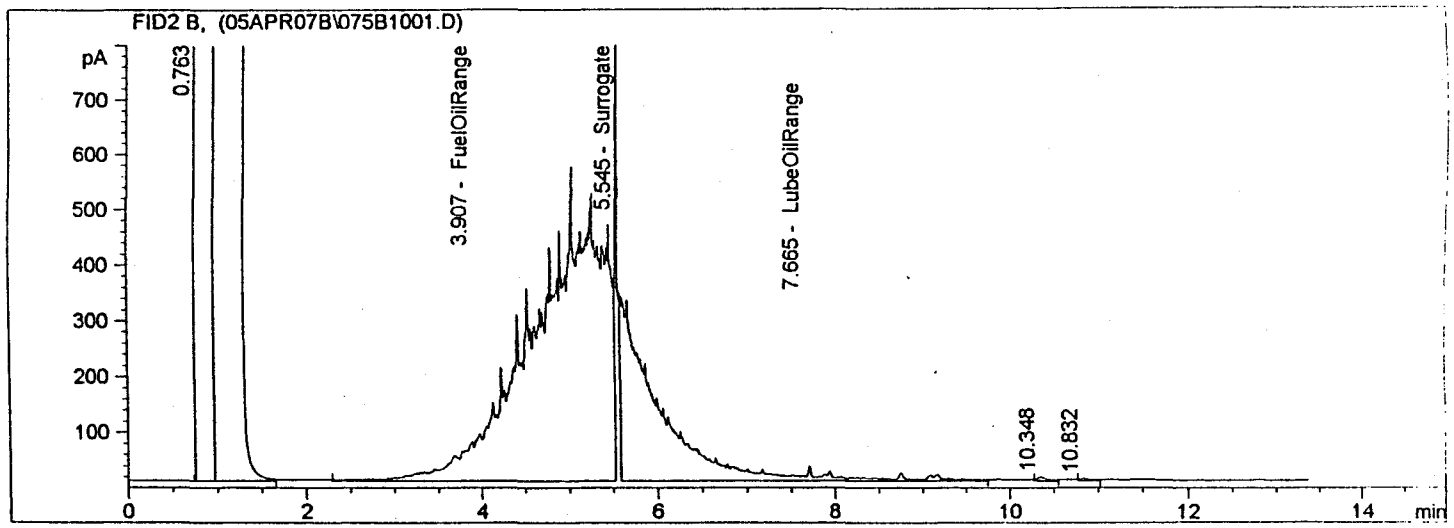
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.907	HHA+	5.22816e4	1785.06948	FuelOilRange
2	5.562	MM	806.30579	71.55322	Surrogate
3	7.667	HHA+	1.73782e4	640.43443	LubeOilRange

Totals: 2497.05713

Results obtained with enhanced integrator!

B.32

Gas chromatogram of treatment system 32 obtained in phase I experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	2.91336e4	994.45656	FuelOilRange
.|	5.545	MM	558.62067	49.57314	Surrogate
.|	7.665	HHA+	1.00594e4	371.75365	LubeOilRange

Totals: 1415.78335

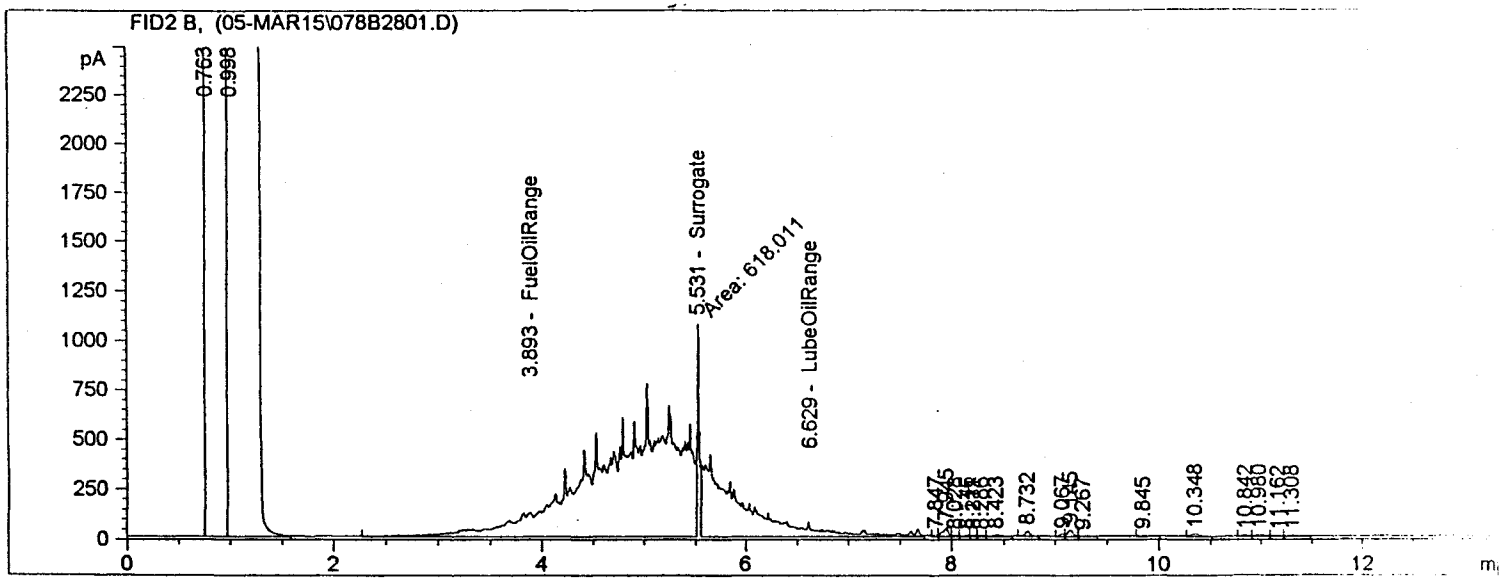
Results obtained with enhanced integrator!

Appendix C

Phase II Biodegradation Experiments

C.1

Gas chromatogram of treatment system 1 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

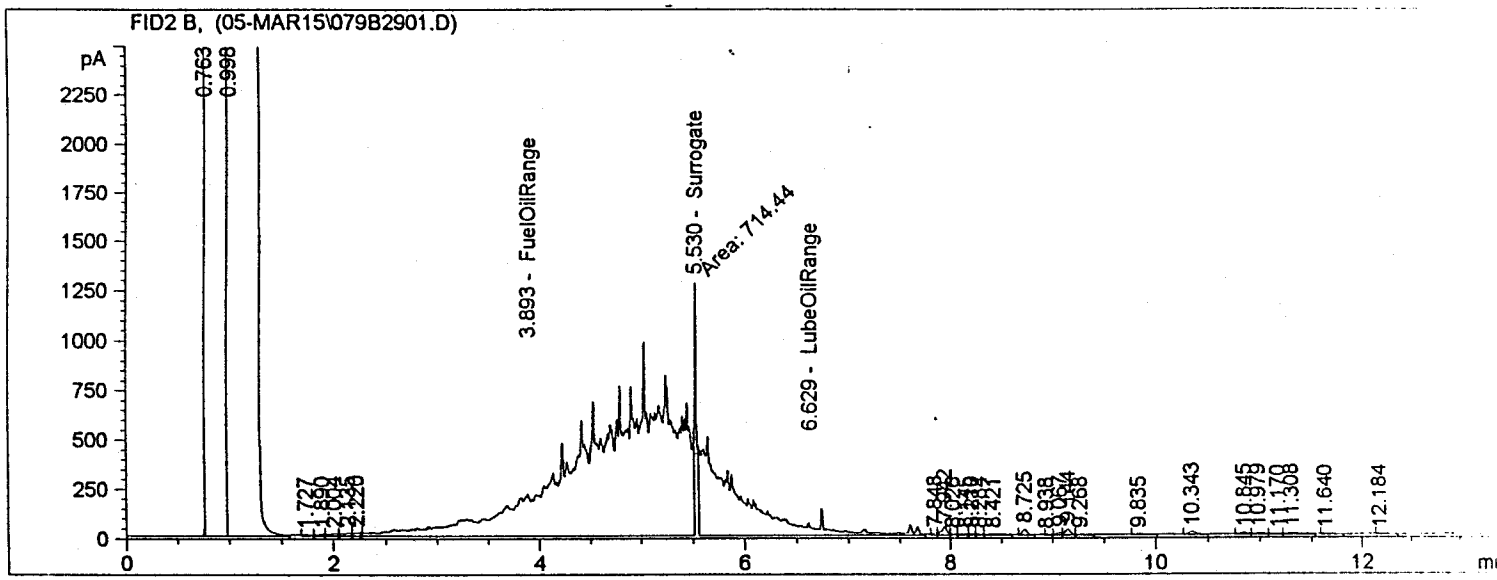
RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	3.70447e4	3.41387e-2	1264.65960		FuelOilRange
5.531	MM	618.01105	6.32391e-2	39.08247		Surrogate
6.629	HHA+	1.07327e4	3.69406e-2	396.47319		LubeOilRange

Totals : 1700.21525

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.2

Gas chromatogram of treatment system 2 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

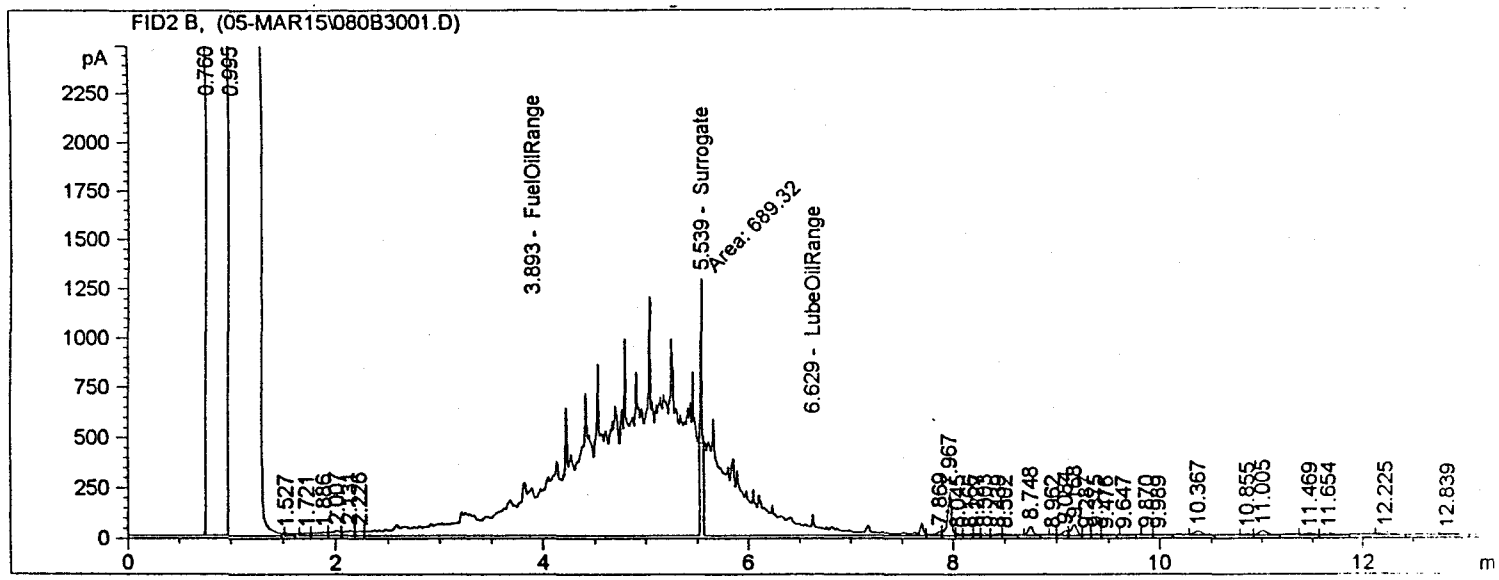
Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	5.19643e4	3.41433e-2	1774.23259		FuelOilRange
5.530	MM	714.44012	6.32391e-2	45.18056		Surrogate
6.629	HHA+	1.28973e4	3.69020e-2	475.93441		LubeOilRange
Totals :				2295.34756		

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.3

Gas chromatogram of treatment system 3 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	5.75067e4	3.41444e-2	1963.52966		FuelOilRange
5.539	MM	689.31952	6.32391e-2	43.59195		Surrogate
6.629	HHA+	1.38520e4	3.68888e-2	510.98526		LubeOilRange

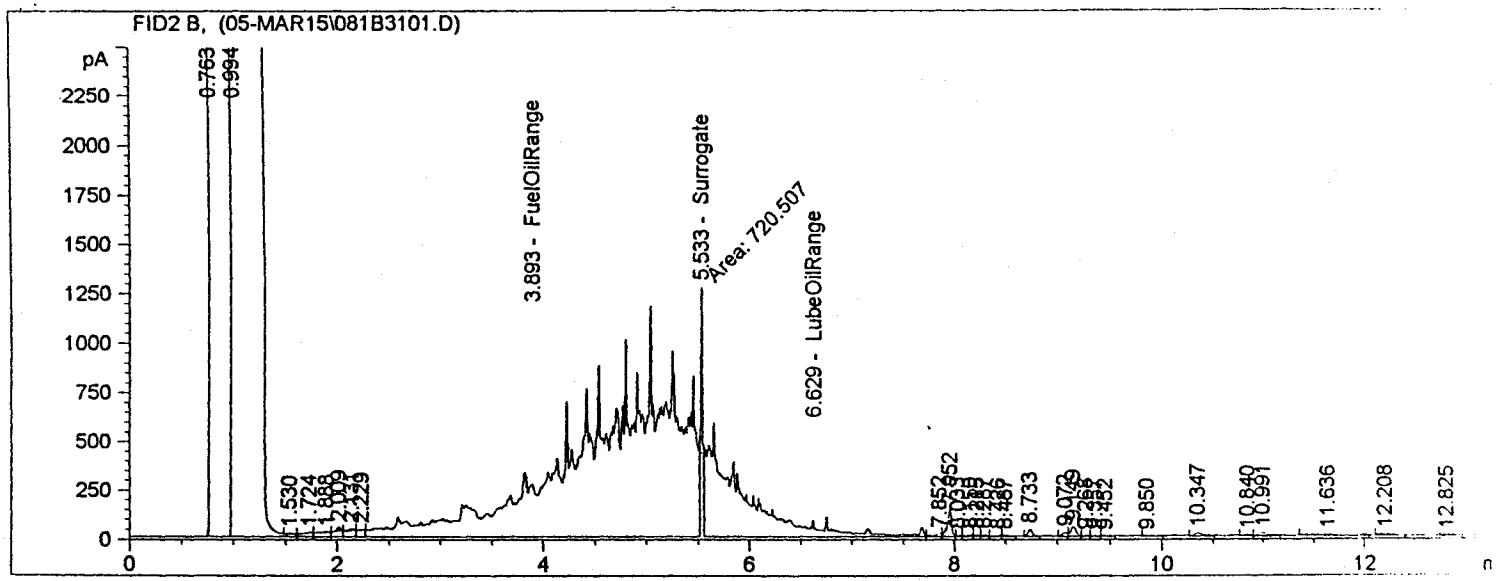
Totals : 2518.10688

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration

C.4

Gas chromatogram of treatment system 4 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	6.01346e4	3.41448e-2	2053.28348		FuelOilRange
5.533	MM	720.50665	6.32391e-2	45.56420		Surrogate
6.629	HHA+	1.32356e4	3.68971e-2	488.35344		LubeOilRange

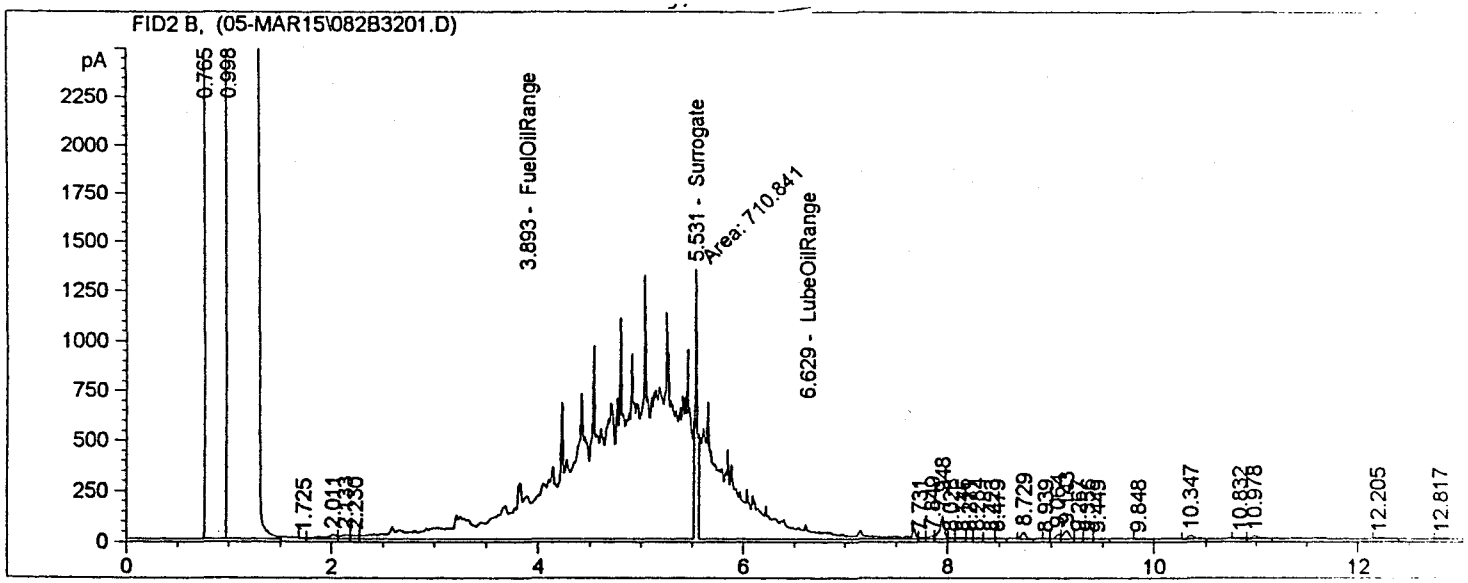
Totals : 2587.20112

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calibration warn

C.5

Gas chromatogram of treatment system 5 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

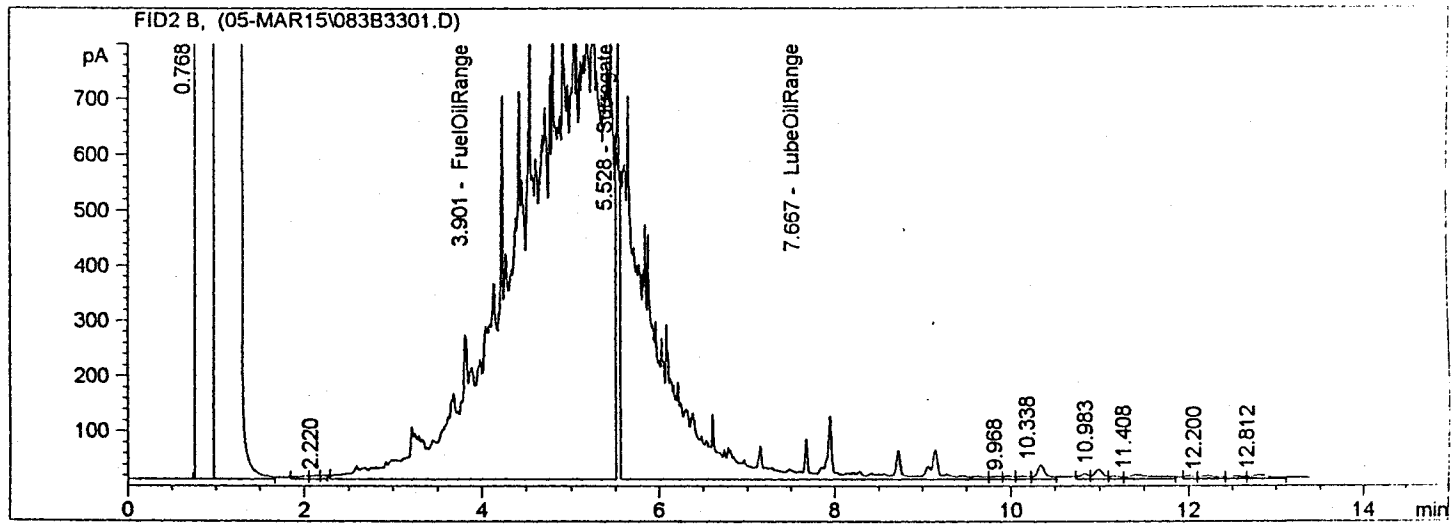
RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	5.92249e4	3.41447e-2	2022.21451		FuelOilRange
5.531	MM	710.84076	6.32391e-2	44.95293		Surrogate
6.629	HHA+	1.43971e4	3.68821e-2	530.99621		LubeOilRange

Totals : 2598.16366

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.6

Gas chromatogram of treatment system 6 obtained in phase II experiments after 45 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Fri, Apr. 15, 2005 3:33:35 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

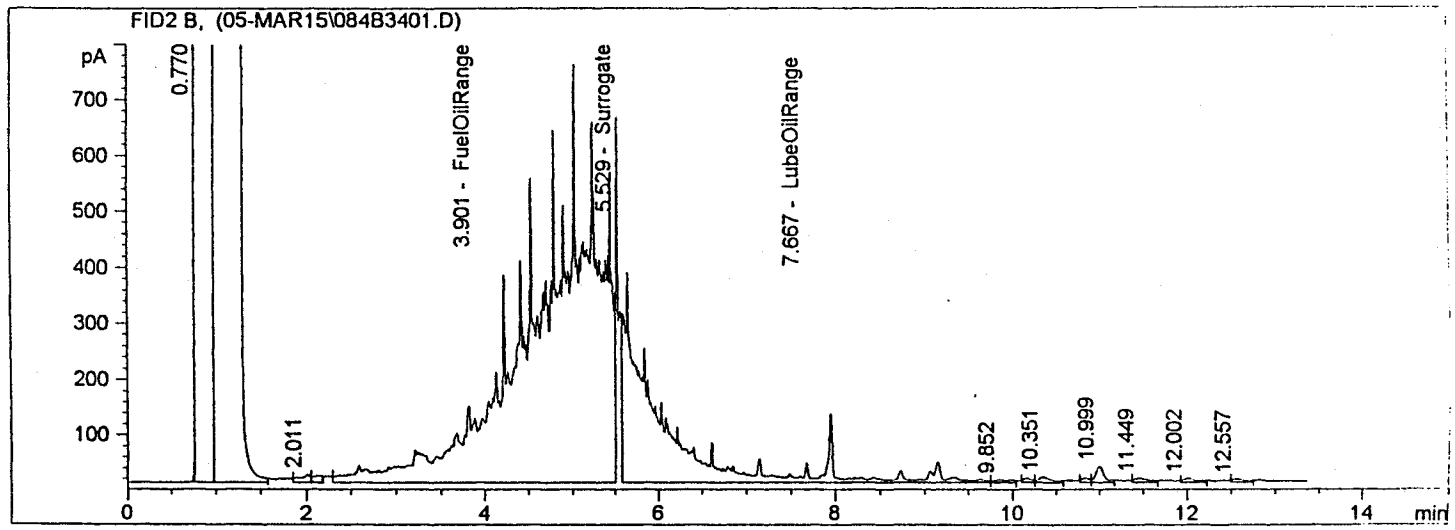
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	5.95625e4	2033.74437	FuelOilRange
.|	5.528	MM	273.10272	24.23569	Surrogate
.|	7.667	HHA+	1.84771e4	680.77453	LubeOilRange

Totals: 2738.75459

Results obtained with enhanced integrator!

C.7

Gas chromatogram of treatment system 7 obtained in phase II experiments after 45 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Fri, Apr. 15, 2005 3:33:35 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

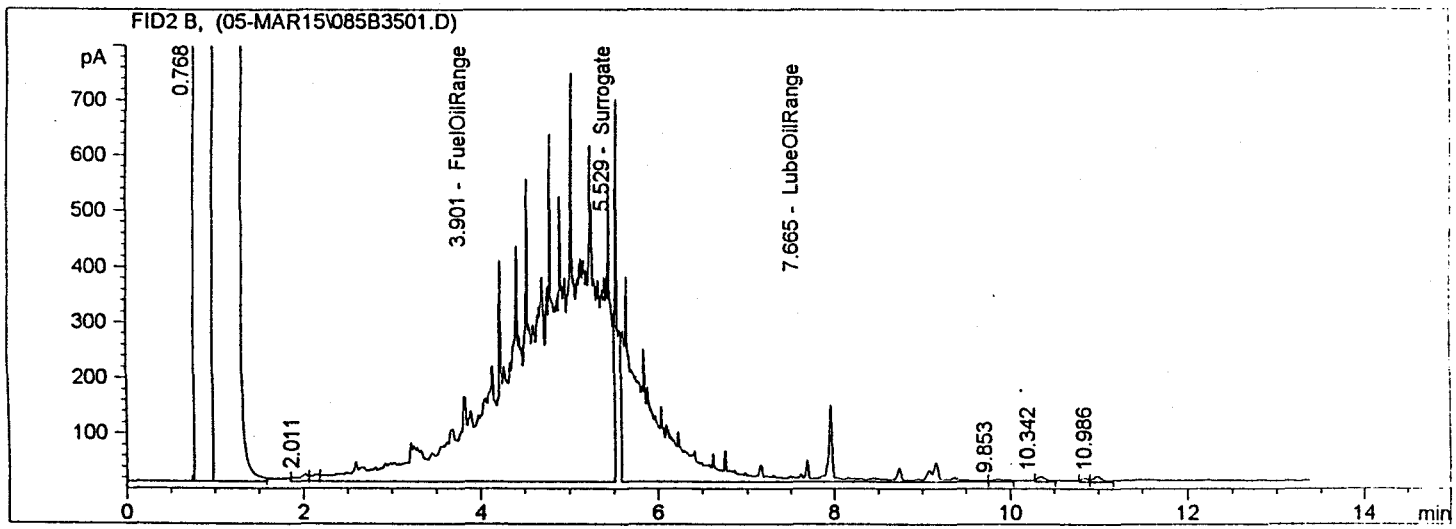
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	3.32557e4	1135.24679	FuelOilRange
.|	5.529	MM	273.90784	24.30714	Surrogate
.|	7.667	HHA+	9687.31934	358.09517	LubeOilRange

Totals: 1517.64910

Results obtained with enhanced integrator!

C.8

Gas chromatogram of treatment system 8 obtained in phase II experiments after 45 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Fri, Apr. 15, 2005 3:33:35 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

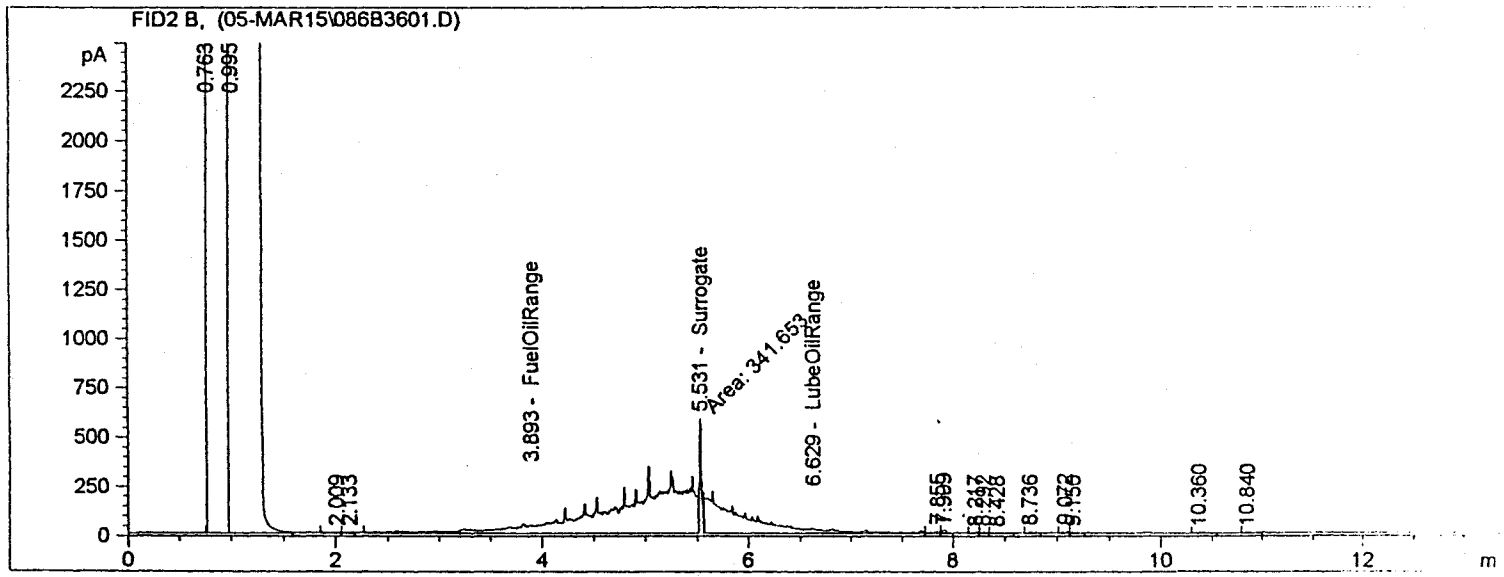
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.901	HHA+	3.21755e4	1098.35159	FuelOilRange
.|	5.529	MM	332.37793	29.49590	Surrogate
.|	7.665	HHA+	8735.92480	323.16876	LubeOilRange

Totals: 1451.01625

Results obtained with enhanced integrator!

C.9

Gas chromatogram of treatment system 9 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

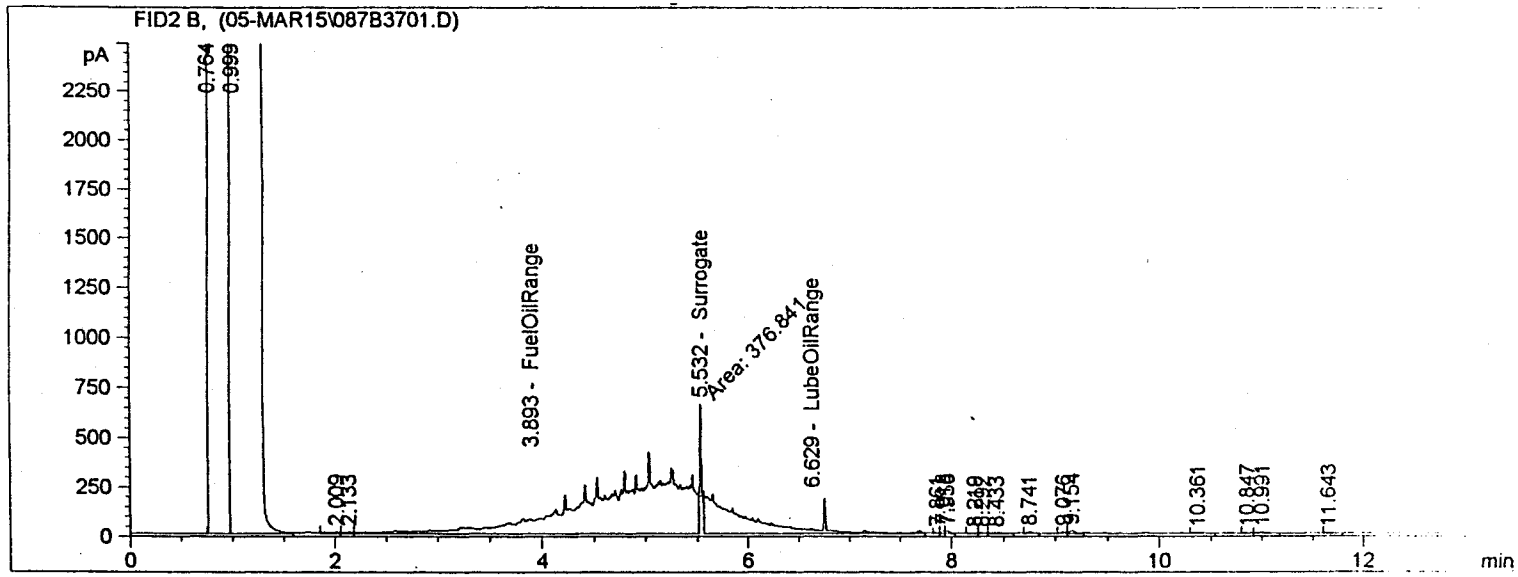
Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	1.44972e4	3.41140e-2	494.55716		FuelOilRange
5.531	MM	341.65314	6.32391e-2	21.60584		Surrogate
6.629	HHA+	5379.70361	3.71692e-2	199.95933		LubeOilRange
Totals :				716.12233		

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.10

Gas chromatogram of treatment system 10 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

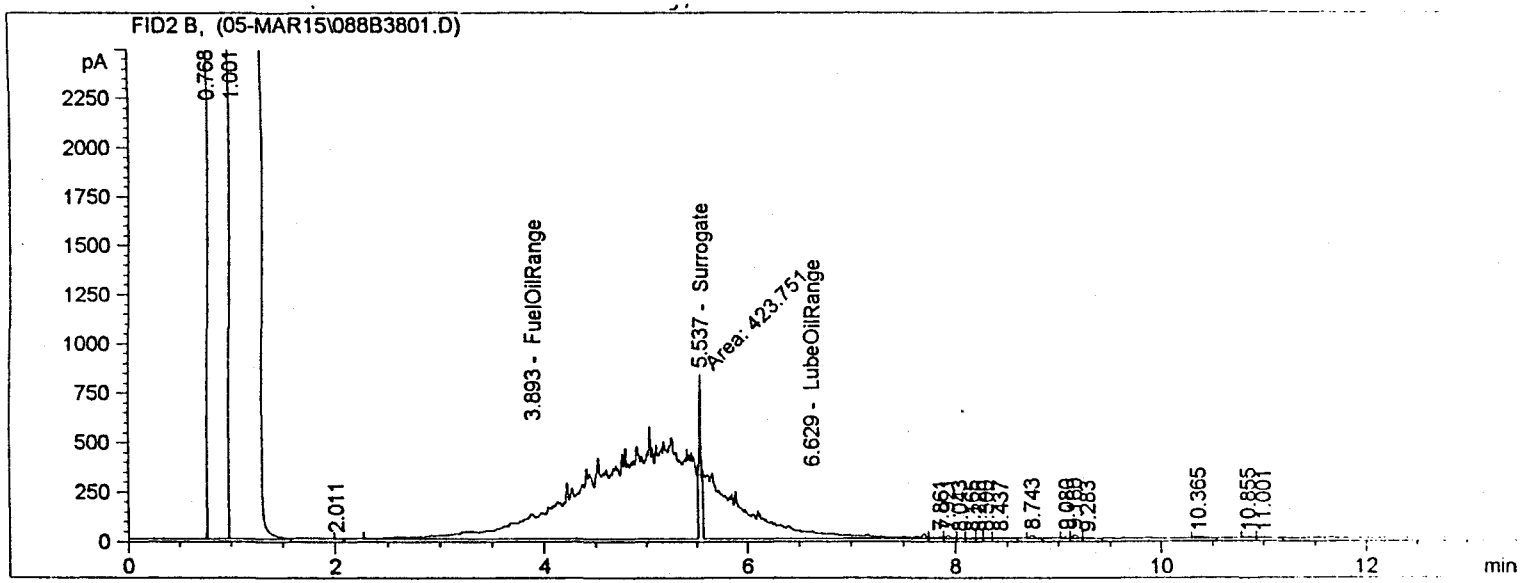
RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	2.09559e4	3.41265e-2	715.15104		FuelOilRange
5.532	MM	376.84134	6.32391e-2	23.83111		Surrogate
6.629	HHA+	5697.29443	3.71437e-2	211.61833		LubeOilRange

Totals : 950.60048

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.11

Gas chromatogram of treatment system 11 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

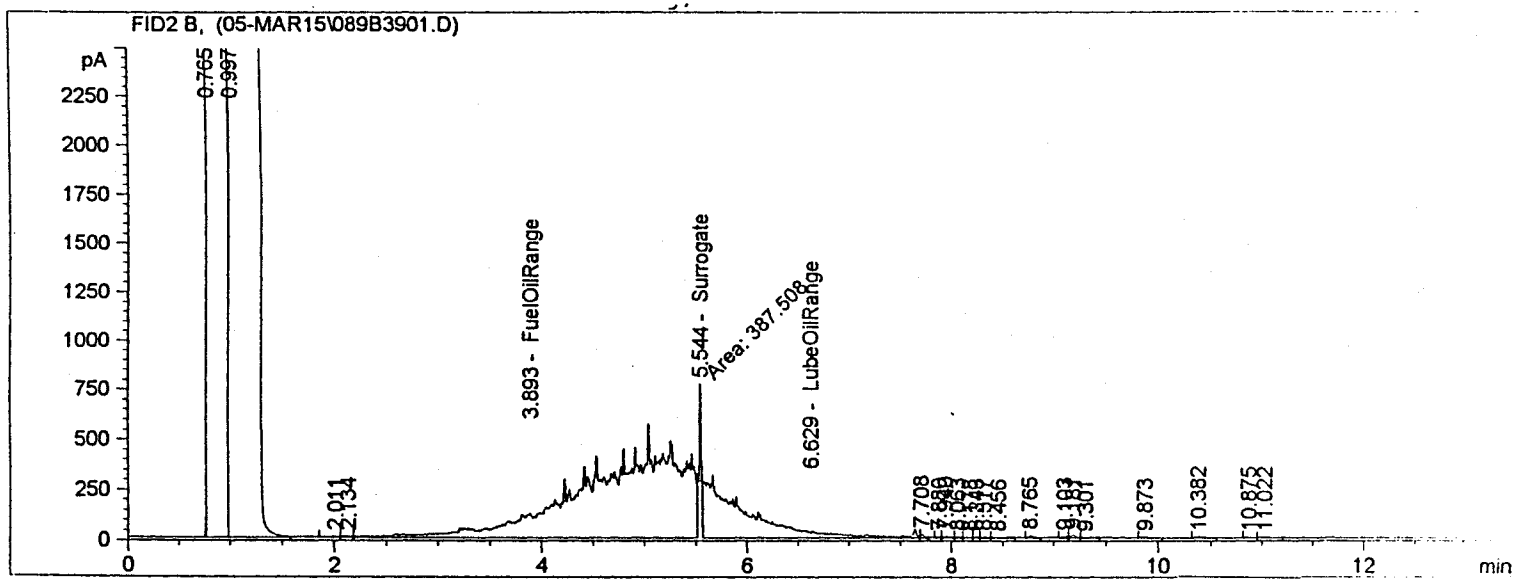
Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	3.48640e4	3.41377e-2	1190.17677		FuelOilRange
5.537	MM	423.75150	6.32391e-2	26.79767		Surrogate
6.629	HHA+	9526.60059	3.69696e-2	352.19507		LubeOilRange
Totals :				1569.16950		

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.12

Gas chromatogram of treatment system 12 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

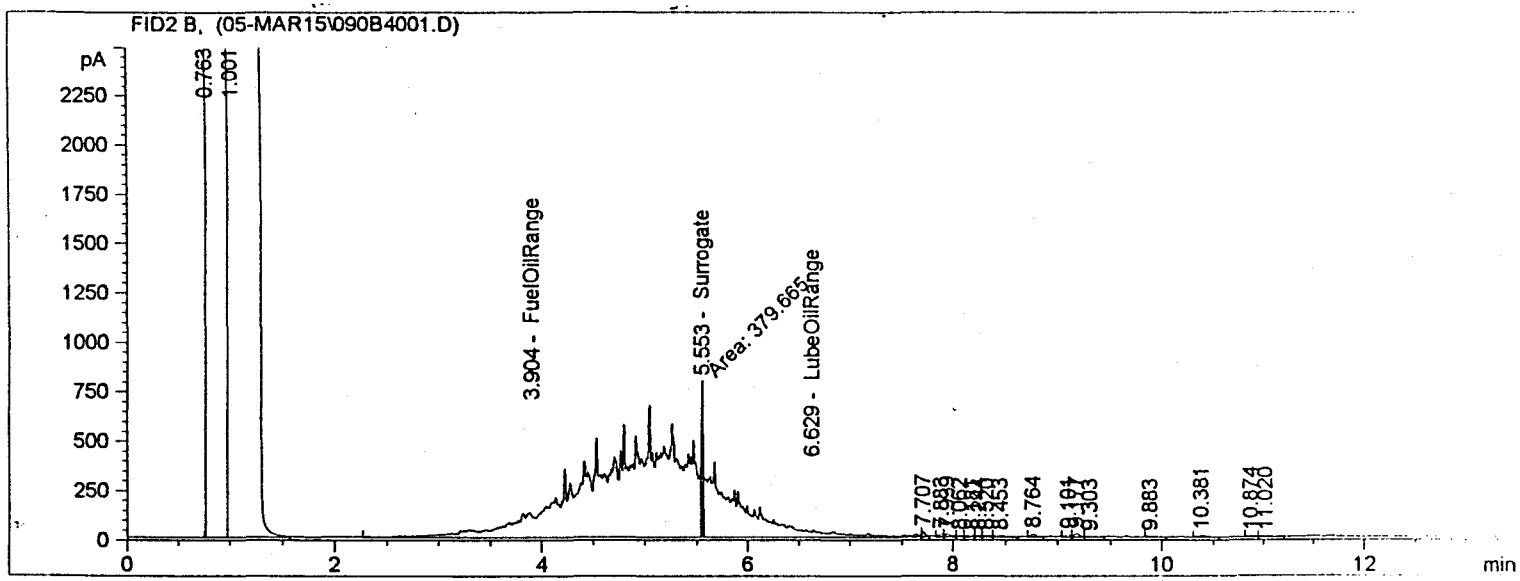
RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	3.13470e4	3.41358e-2	1070.05455		FuelOilRange
5.544	MM	387.50760	6.32391e-2	24.50563		Surrogate
6.629	HHA+	8612.79980	3.69971e-2	318.64875		LubeOilRange

Totals : 1413.20893

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.13

Gas chromatogram of treatment system 13 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

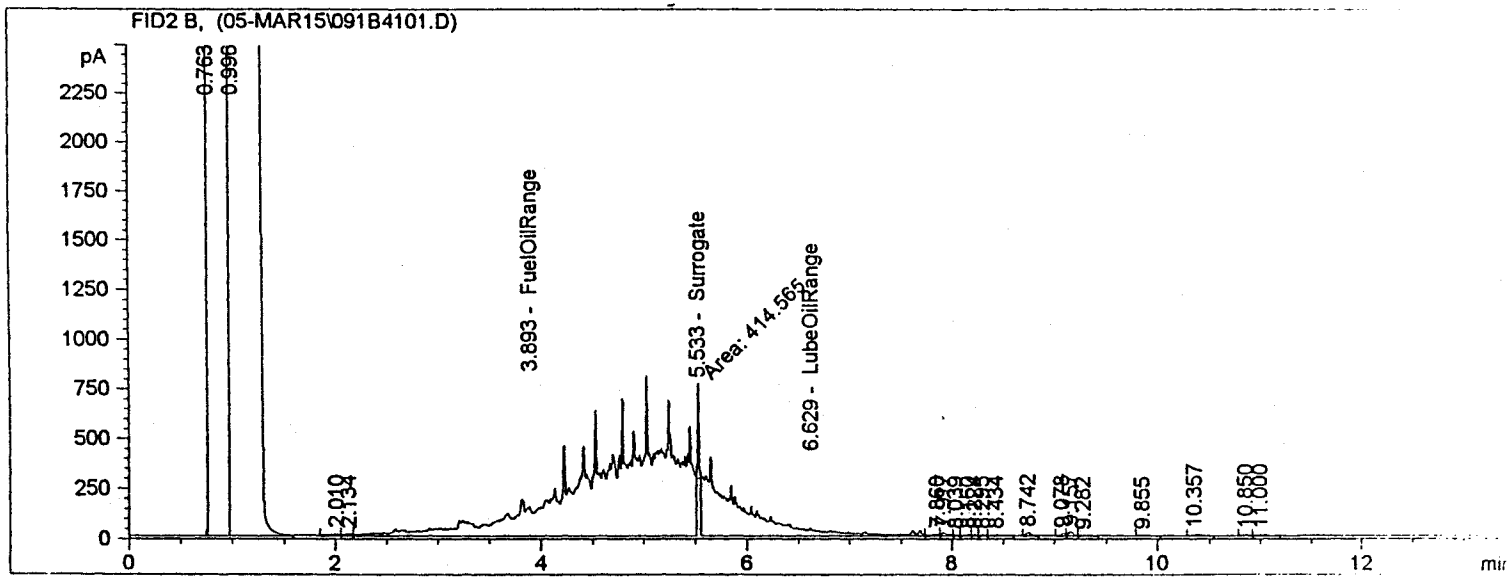
Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.904	HHA+	3.40798e4	3.41373e-2	1163.39327		FuelOilRange
5.553	MM	379.66510	6.32391e-2	24.00968		Surrogate
6.629	HHA+	9219.54590	3.69783e-2	340.92286		LubeOilRange
Totals :				1528.32581		

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.14

Gas chromatogram of treatment system 14 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

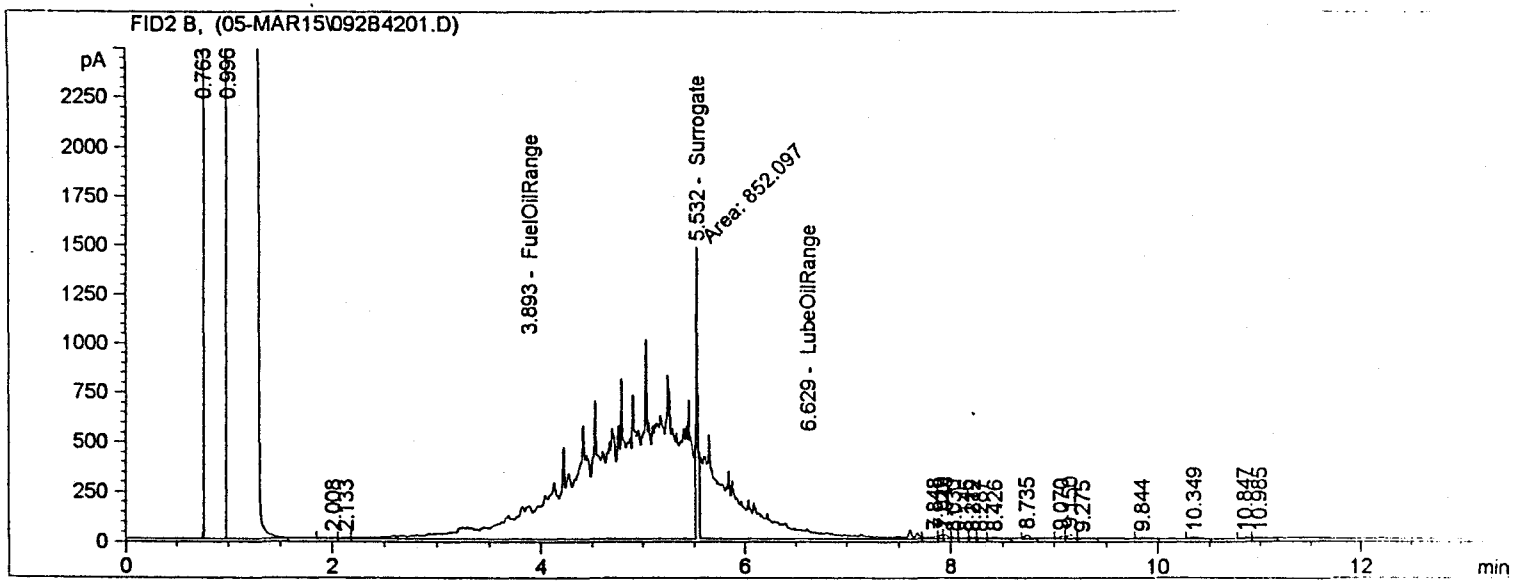
RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	3.63738e4	3.41384e-2	1241.74238		FuelOilRange
5.533	MM	414.56519	6.32391e-2	26.21673		Surrogate
6.629	HHA+	8961.66504	3.69860e-2	331.45585		LubeOilRange

Totals : 1599.41496

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.15

Gas chromatogram of treatment system 15 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	4.60866e4	3.41418e-2	1573.48225		FuelOilRange
5.532	MM	852.09692	6.32391e-2	53.88585		Surrogate
6.629	HHA+	1.21309e4	3.69141e-2	447.80239		LubeOilRange

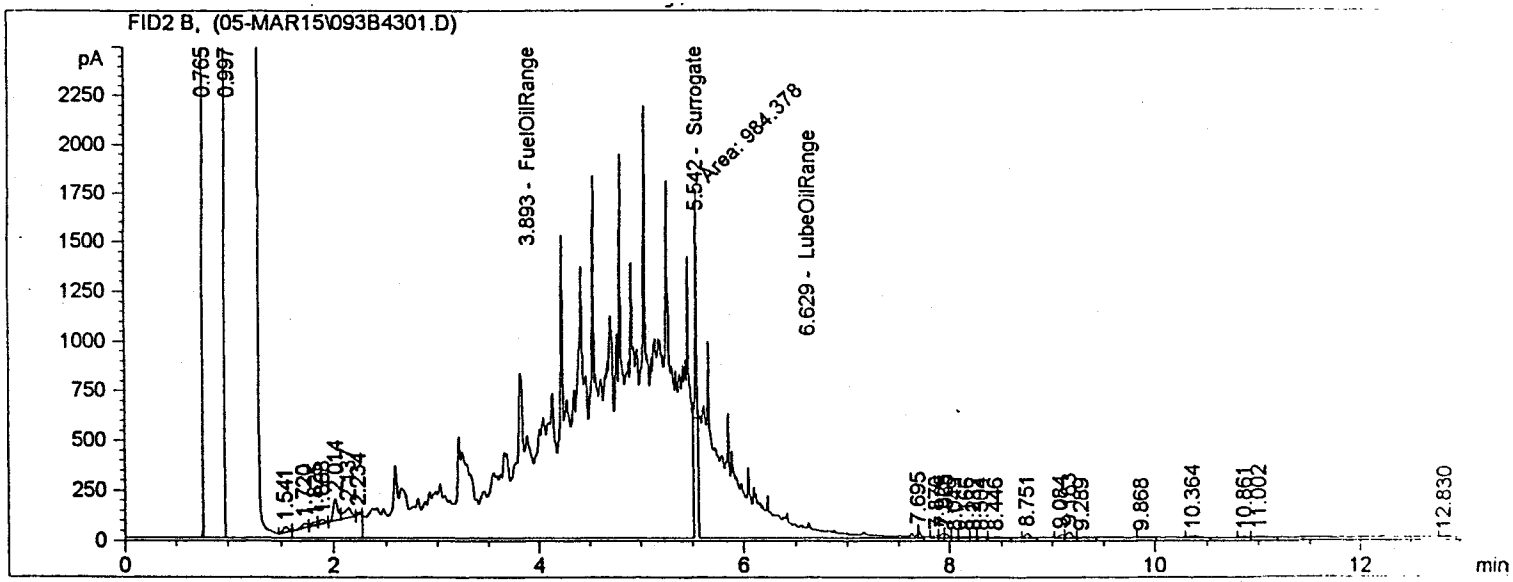
Totals : 2075.17049

Results obtained with enhanced integrator!
1 Warnings or Errors :

Warning : Calib...

C.16

Gas chromatogram of treatment system 16 obtained in phase II experiments after 45 days



==========

External Standard Report

==========

Sorted By : Signal
Calib. Data Modified : 05/03/16 3:48:57 PM
Multiplier : 1.0000
Dilution : 1.0000

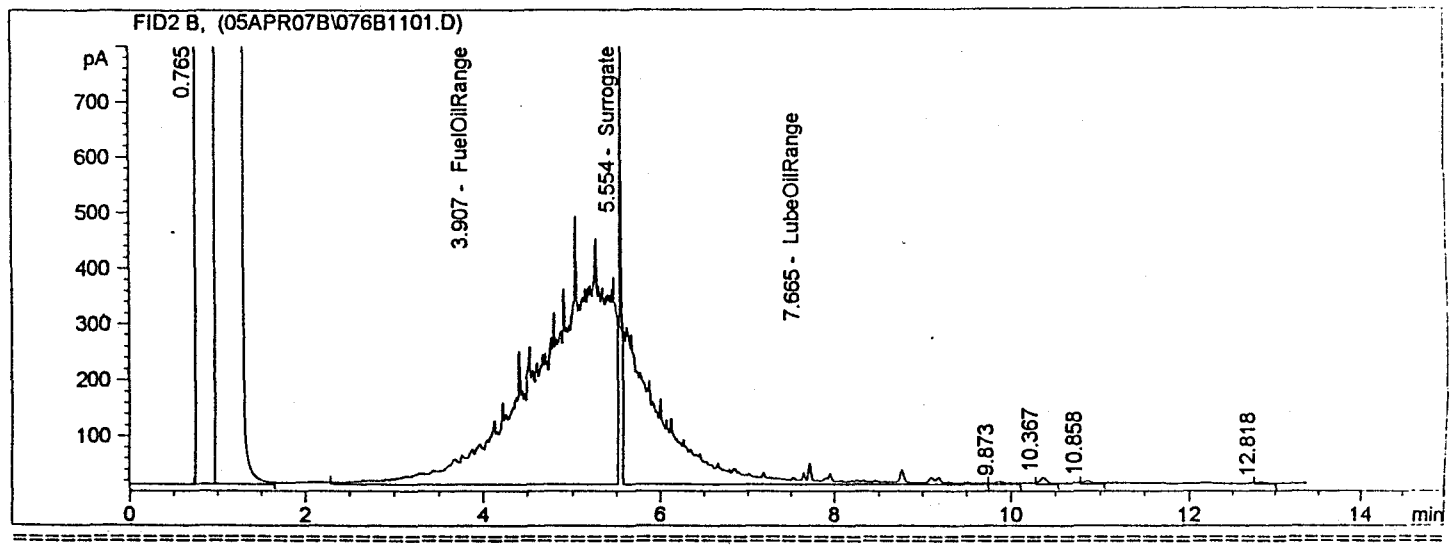
Signal 1: FID2 B,

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [ug/mL]	Grp	Name
3.893	HHA+	1.03183e5	3.41489e-2	3523.58872		FuelOilRange
5.542	MM	984.37775	6.32391e-2	62.25117		Surrogate
6.629	HHA+	1.75633e4	3.68512e-2	647.22979		LubeOilRange
Totals :				4233.06968		

Results obtained with enhanced integrator!
1 Warnings or Errors :

C.17

Gas chromatogram of treatment system 17 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

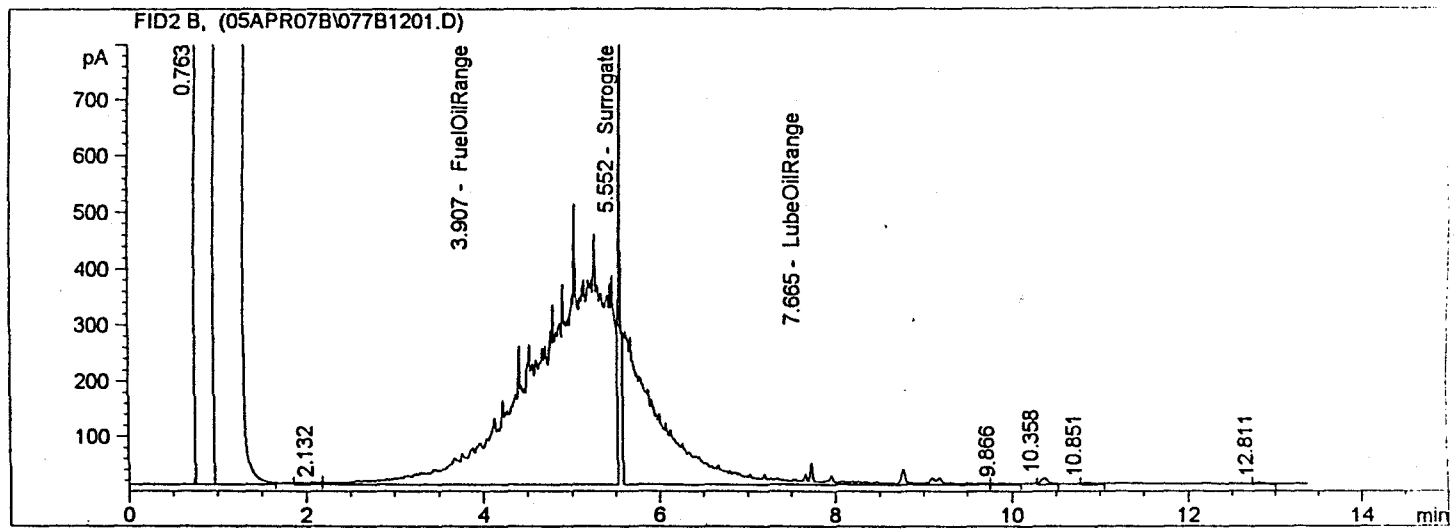
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name	
.|	3.907	HHA+	2.36709e4	807.88118	FuelOilRange	|.
.|	5.554	MM	603.08716	53.51919	Surrogate	|.
.|	7.665	HHA+	9255.89258	342.25717	LubeOilRange	|.

Totals: 1203.65754

Results obtained with enhanced integrator!

C.18

Gas chromatogram of treatment system 18 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

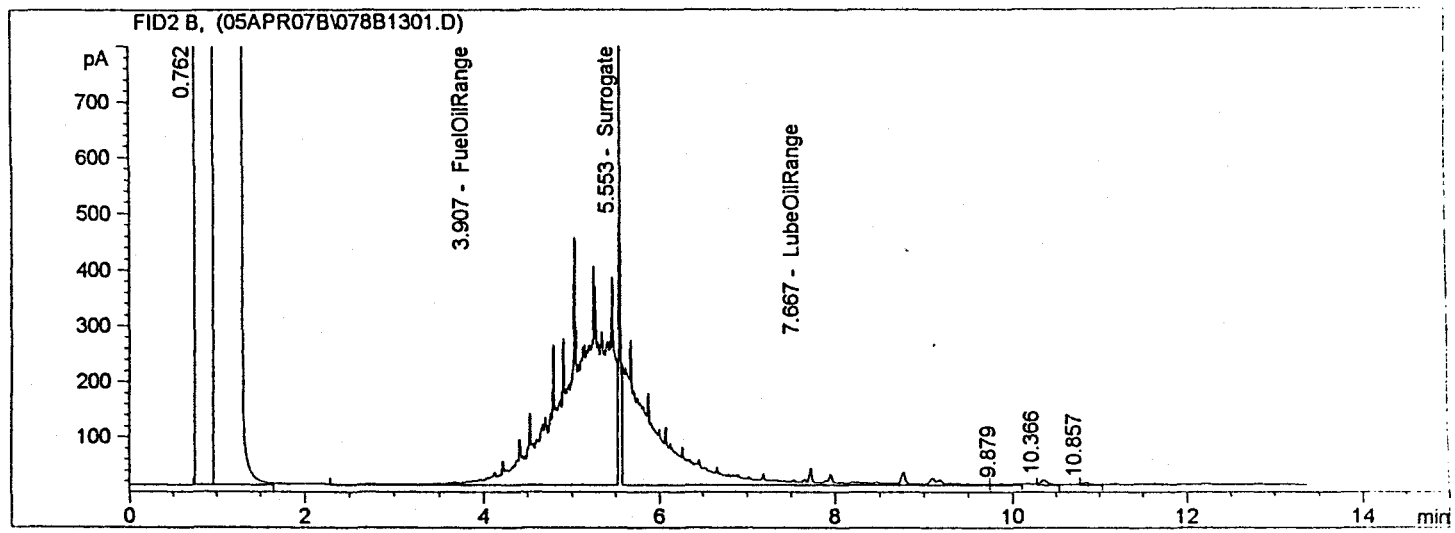
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
	3.907	HHA+	2.46273e4	840.54634	FuelOilRange
	5.552	MM	589.45190	52.30917	Surrogate
	7.665	HHA+	8843.31543	327.11115	LubeOilRange

Totals: 1219.96666

Results obtained with enhanced integrator!

C.19

Gas chromatogram of treatment system 19 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

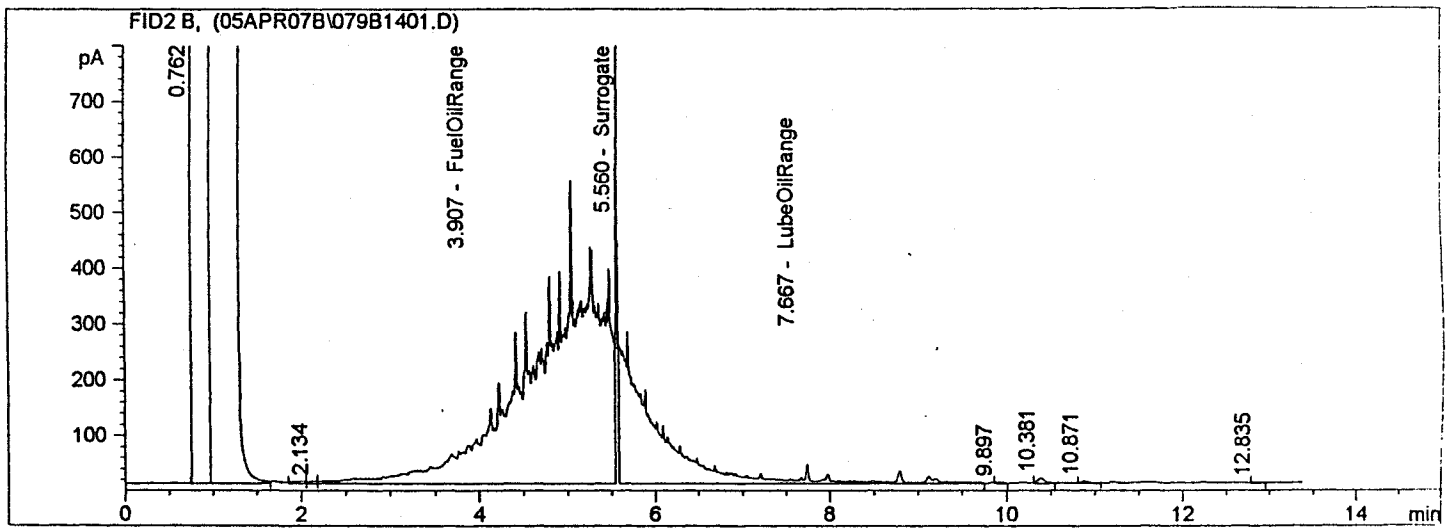
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	1.29771e4	442.63819	FuelOilRange
.|	5.553	MM	507.05603	44.99719	Surrogate
.|	7.667	HHA+	6995.73975	259.28525	LubeOilRange

Totals: 746.92063

Results obtained with enhanced integrator!

C.20

Gas chromatogram of treatment system 20 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

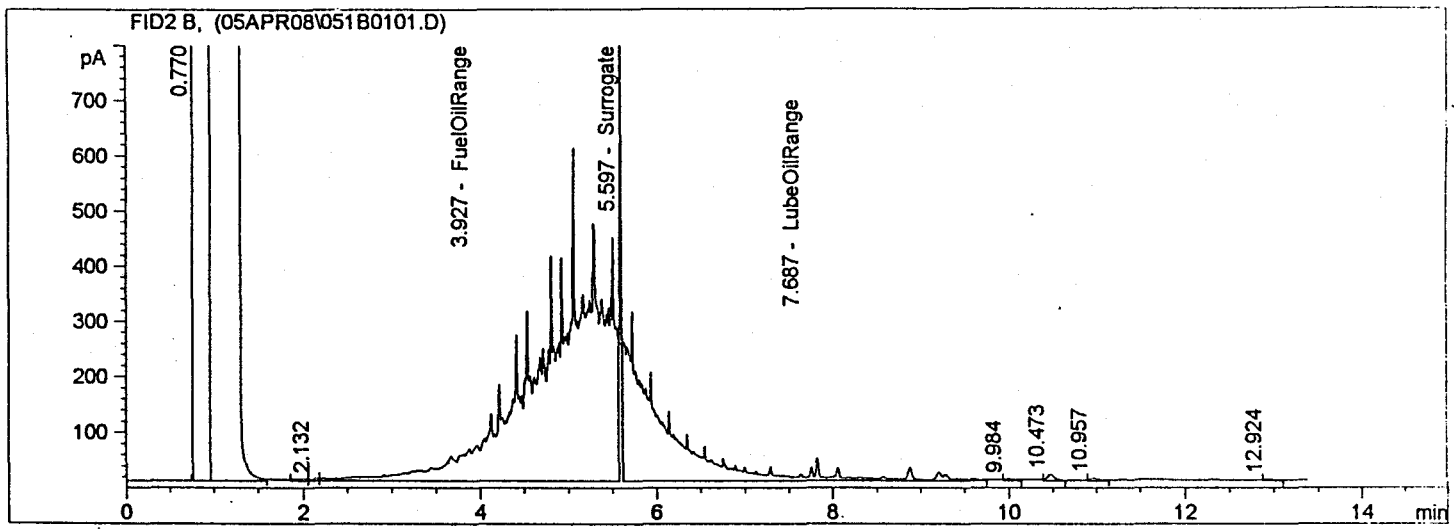
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	2.39044e4	815.85589	FuelOilRange
.|	5.560	MM	558.91254	49.59904	Surrogate
.|	7.667	HHA+	7751.14209	287.01664	LubeOilRange

Totals: 1152.47156

Results obtained with enhanced integrator!

C.21

Gas chromatogram of treatment system 21 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

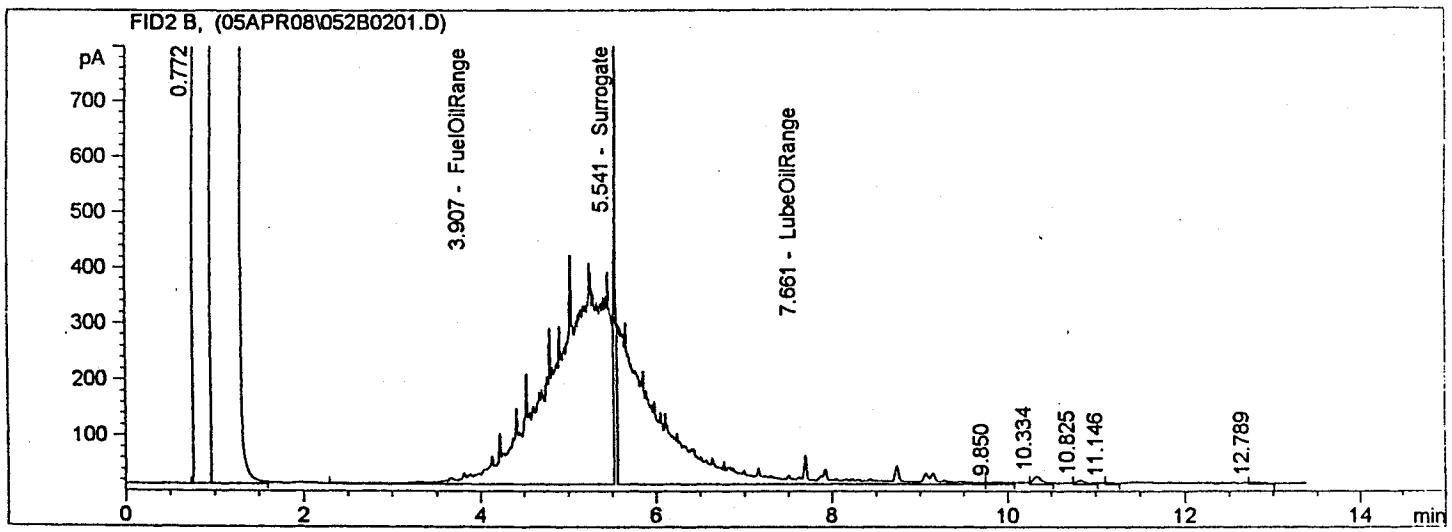
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.927	HHA+	2.29683e4	783.88429	FuelOilRange
.|	5.597	MM	609.08624	54.05156	Surrogate
.|	7.687	HHA+	8537.50879	315.88476	LubeOilRange

Totals: 1153.82060

Results obtained with enhanced integrator!

C.22

Gas chromatogram of treatment system 22 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

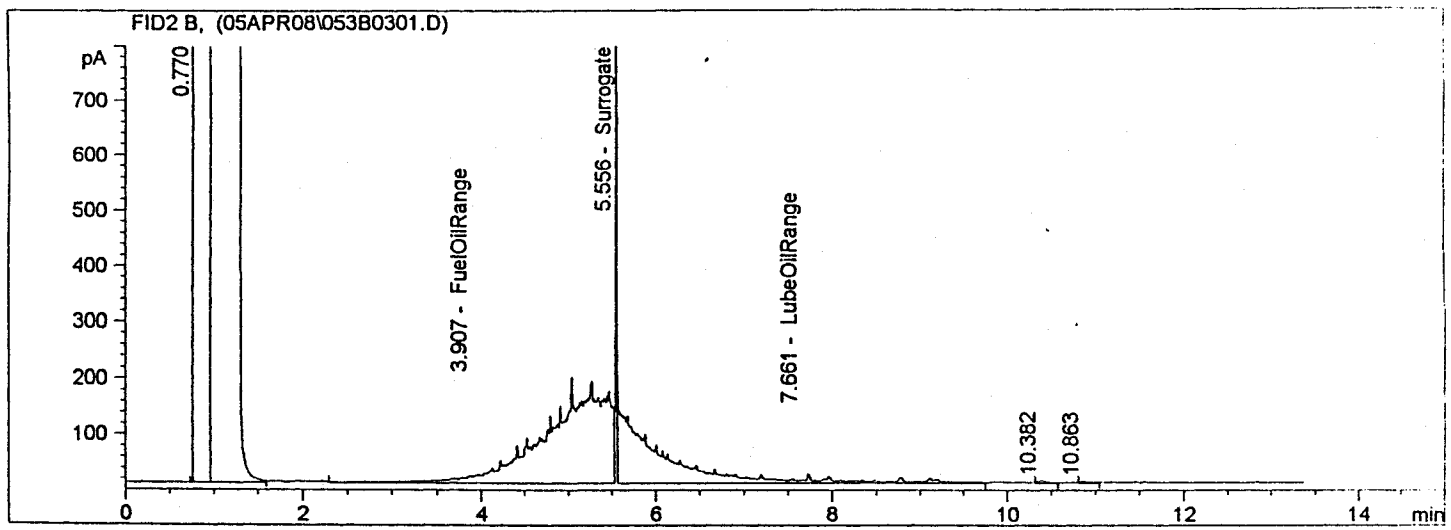
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
	3.907	HHA+	1.74847e4	596.59460	FuelOilRange
	5.541	MM	561.93243	49.86703	Surrogate
	7.661	HHA+	1.00539e4	371.55372	LubeOilRange

Totals: 1018.01535

Results obtained with enhanced integrator!

C.23

Gas chromatogram of treatment system 23 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

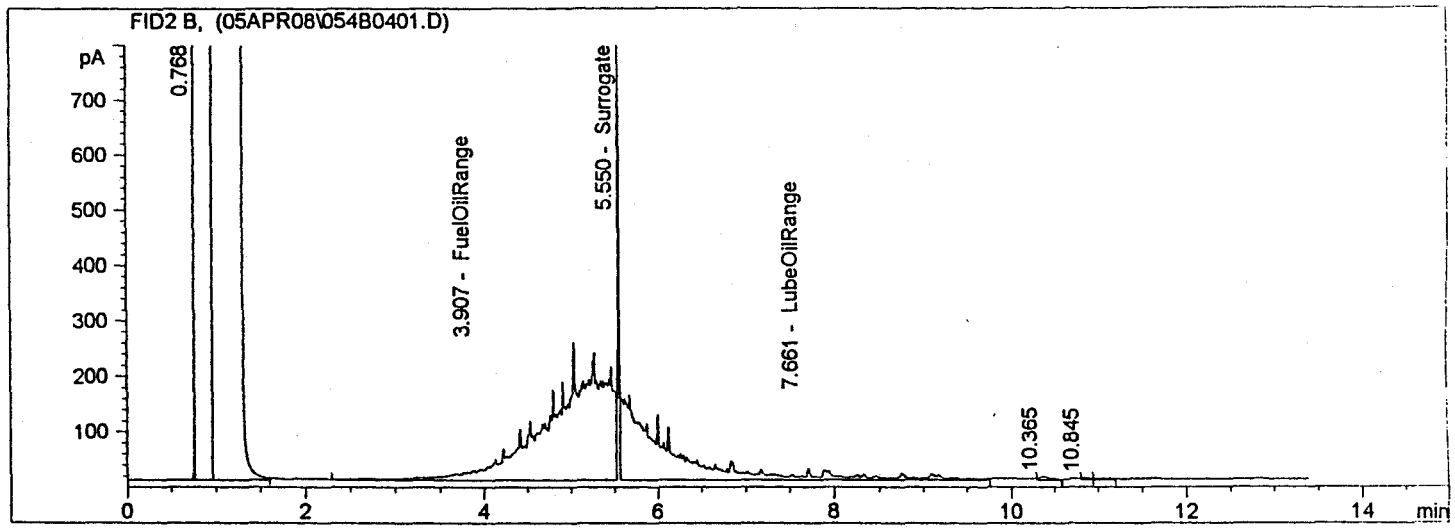
Signal Description : FID2 B,

	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	8811.81250	300.37461	FuelOilRange
.|	5.556	MM	608.33319	53.98473	Surrogate
.|	7.661	HHA+	4728.56494	176.05554	LubeOilRange
	Totals:			530.41488	

Results obtained with enhanced integrator!

C.24

Gas chromatogram of treatment system 24 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

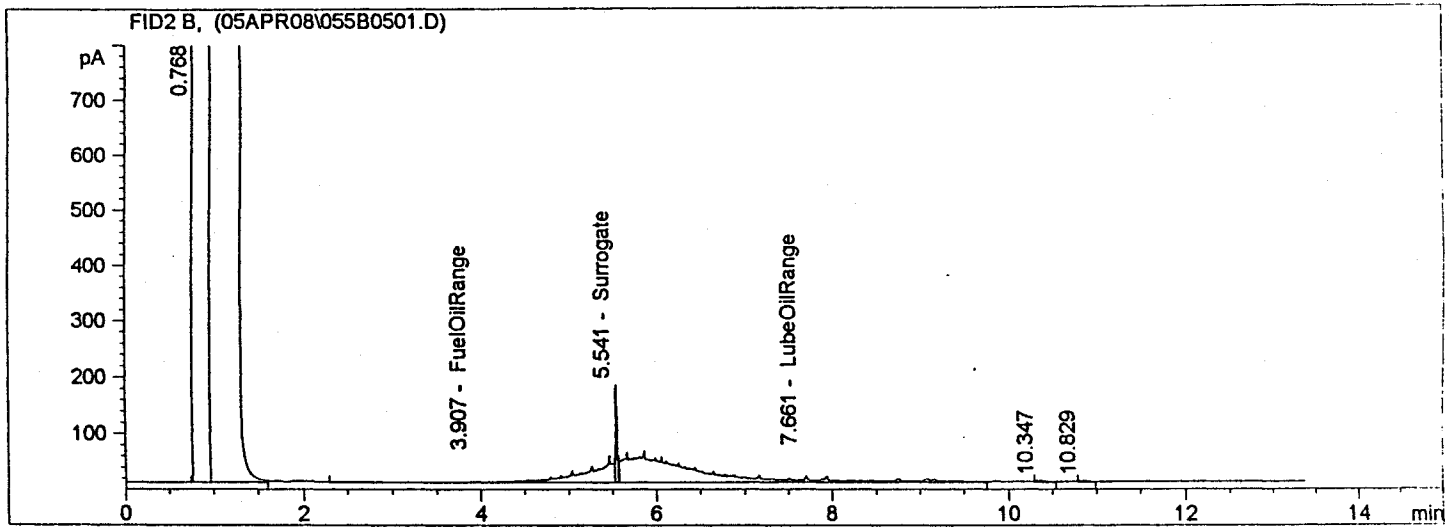
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name	
.|	3.907	HHA+	1.05448e4	359.56481	FuelOilRange	|.
.|	5.550	MM	544.50507	48.32049	Surrogate	|.
.|	7.661	HHA+	5581.85010	207.38028	LubeOilRange	|.

Totals: 615.26558

Results obtained with enhanced integrator!

C.25

Gas chromatogram of treatment system 25 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

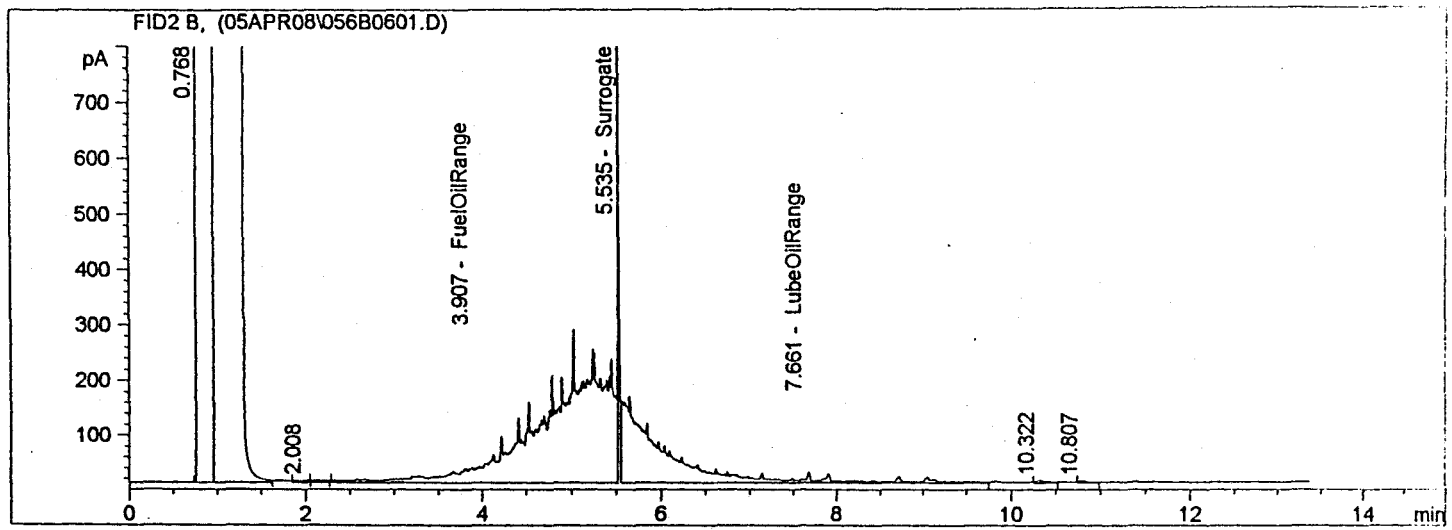
Signal Description : FID2 B,

	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.907	HHA+	1022.89203	34.34695	FuelOilRange
2	5.541	HH	207.30043	18.39626	Surrogate
3	7.661	HHA+	2968.16870	111.43006	LubeOilRange
	Totals:			164.17327	

Results obtained with enhanced integrator!

C.26

Gas chromatogram of treatment system 26 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

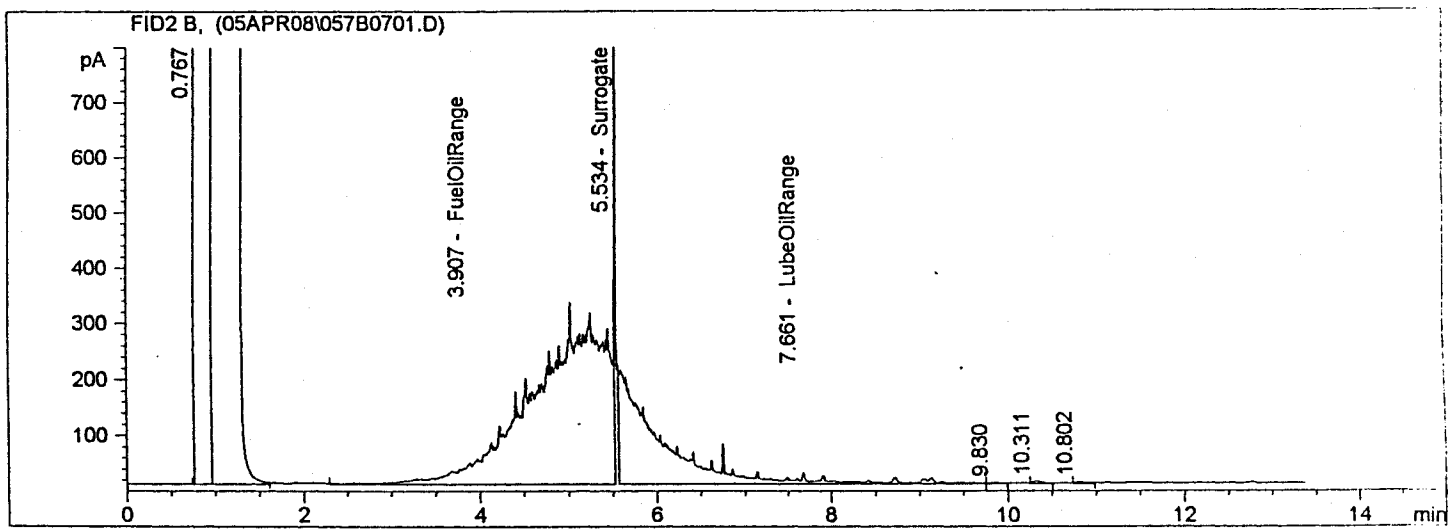
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	1.21236e4	413.48765	FuelOilRange
.|	5.535	MM	661.11121	58.66836	Surrogate
.|	7.661	HHA+	5038.17529	187.42157	LubeOilRange

Totals: 659.57758

Results obtained with enhanced integrator!

C.27

Gas chromatogram of treatment system 27 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

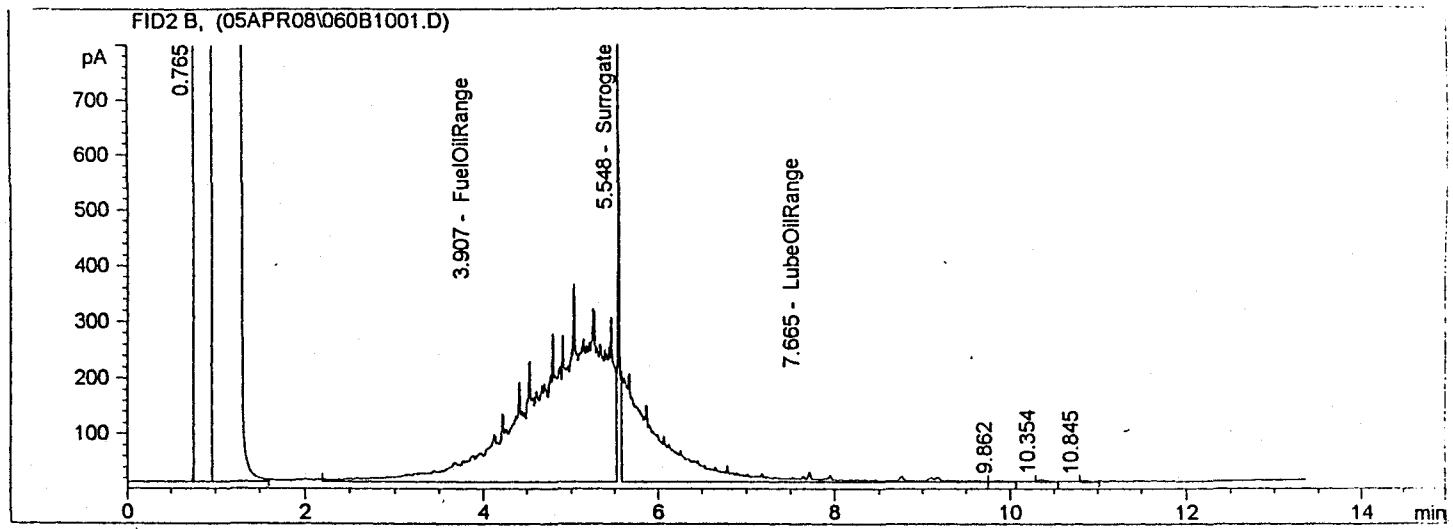
Signal Description : FID2 B,

R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
3.907	HHA+	1.73945e4	593.51448	FuelOilRange
5.534	MM	616.32678	54.69410	Surrogate
7.661	HHA+	6695.06445	248.24723	LubeOilRange
Totals:			896.45581	

Results obtained with enhanced integrator!

C.30

Gas chromatogram of treatment system 30 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

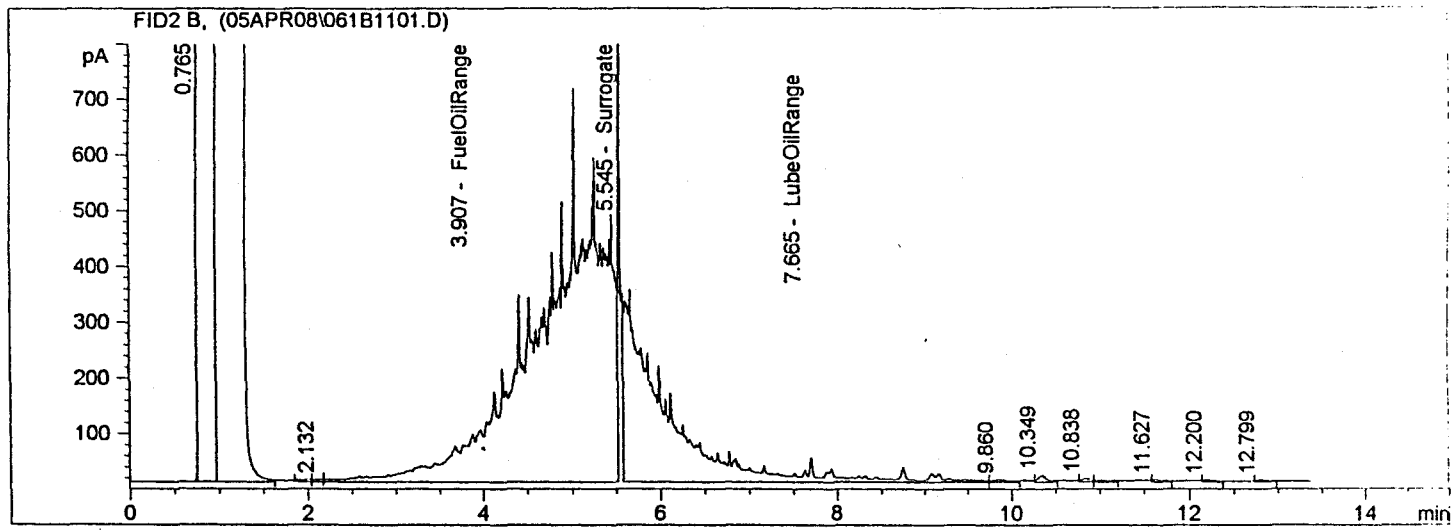
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	1.74676e4	596.00823	FuelOilRange
.|	5.548	MM	631.96454	56.08183	Surrogate
.|	7.665	HHA+	6149.23535	228.20943	LubeOilRange

Totals: 880.29949

Results obtained with enhanced integrator!

C.31

Gas chromatogram of treatment system 31 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

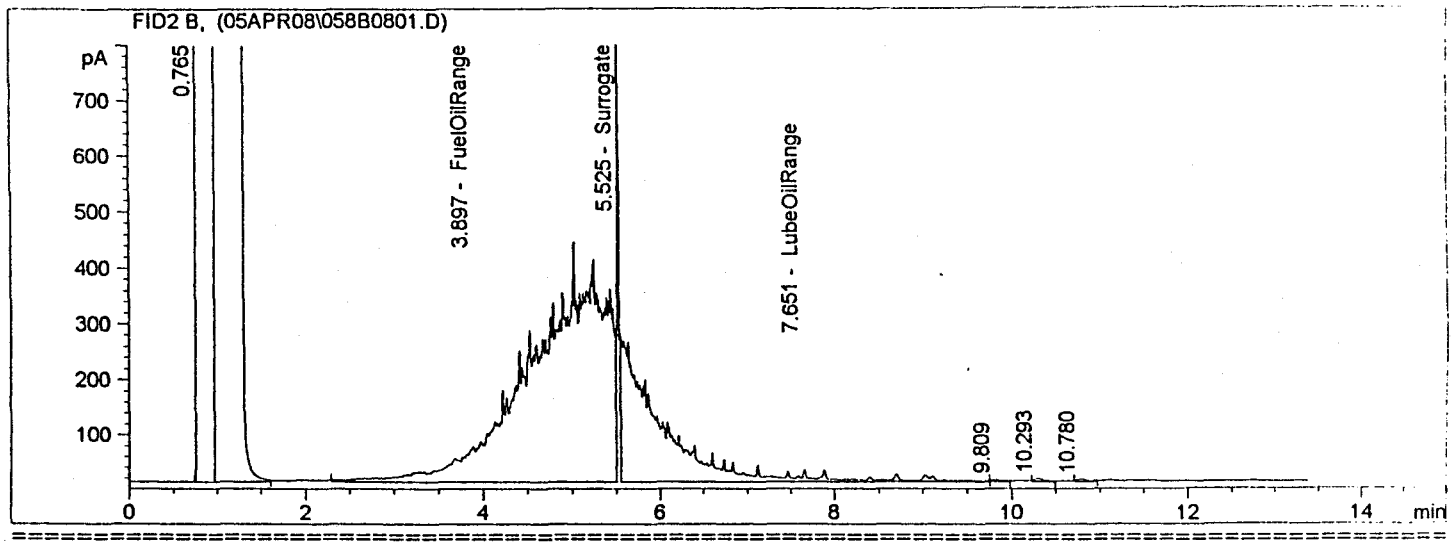
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
.|	3.907	HHA+	3.01123e4	1027.88573	FuelOilRange
.|	5.545	MM	692.85797	61.48563	Surrogate
.|	7.665	HHA+	1.11495e4	411.77172	LubeOilRange

Totals: 1501.14308

Results obtained with enhanced integrator!

C.28

Gas chromatogram of treatment system 28 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

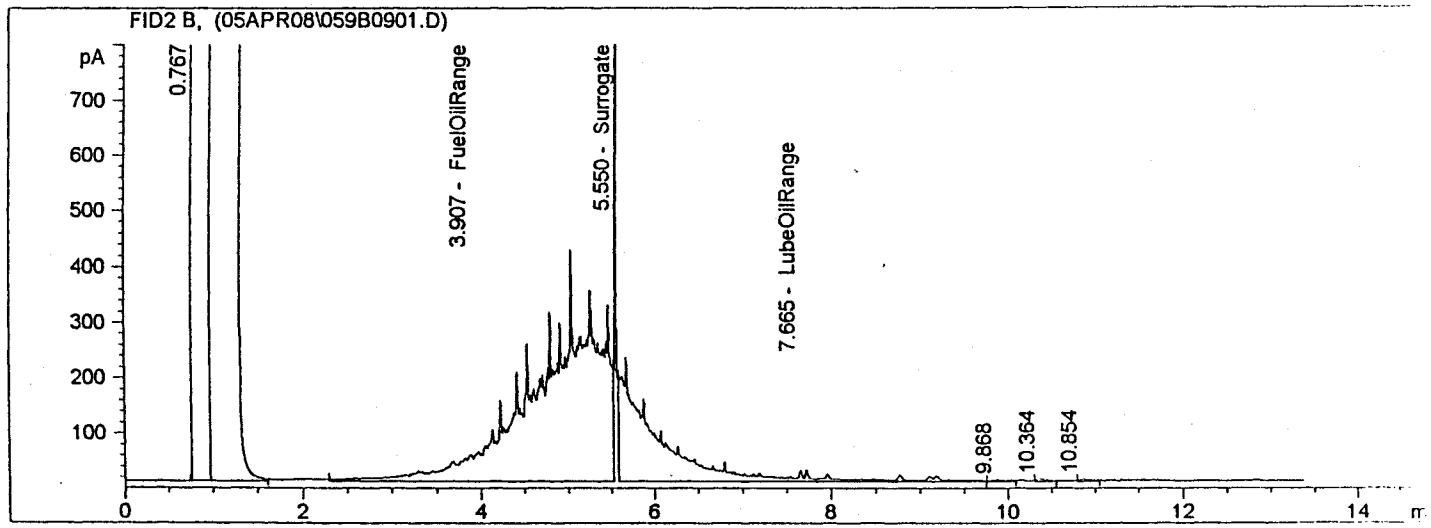
	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.897	HHA+	2.38682e4	814.61945	FuelOilRange
2	5.525	MM	629.96881	55.90472	Surrogate
3	7.651	HHA+	8398.65723	310.78741	LubeOilRange

Totals: 1181.31158

Results obtained with enhanced integrator!

C.29

Gas chromatogram of treatment system 29 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

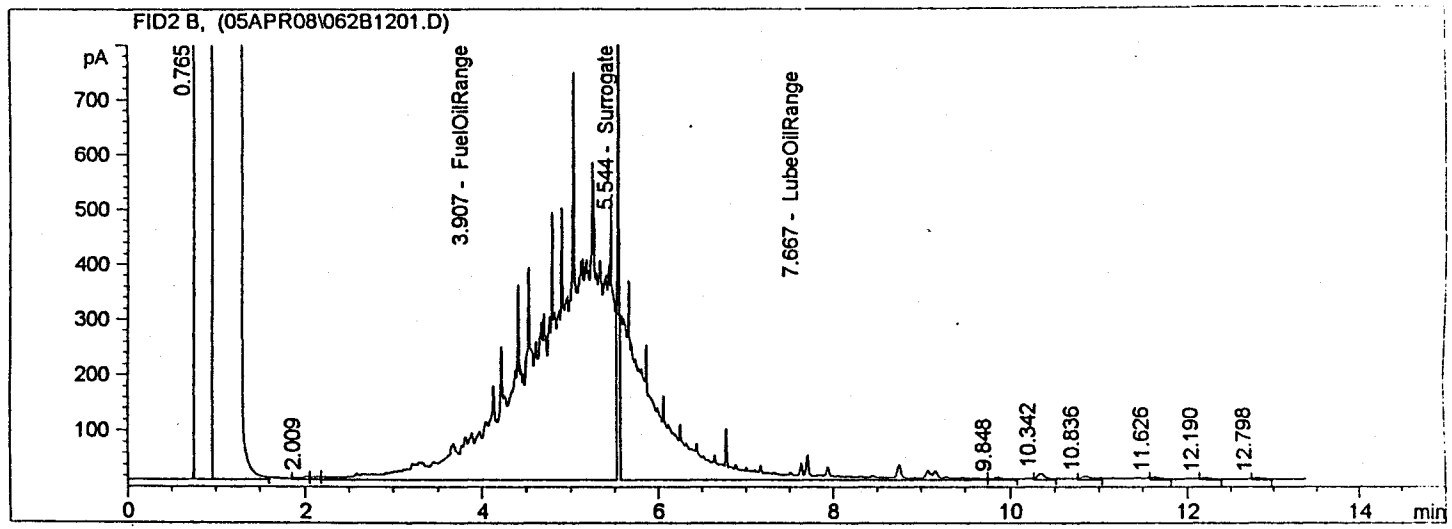
Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

	R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
1	3.907	HHA+	1.81392e4	618.94740	FuelOilRange
2	5.550	MM	661.47540	58.70068	Surrogate
3	7.665	HHA+	6562.77002	243.39060	LubeOilRange
Totals:				921.03868	

C.32

Gas chromatogram of treatment system 32 obtained in phase II experiments after 90 days



Customized Report: Max-nugen

Sorted By : Signal
Calib. Data Modified : Thu, Apr. 14, 2005 8:26:34 pm
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal Description : FID2 B,

R.T. [min]	Type	Area	Amount [ug/mL]	CMP Name
3.907	HHA+	2.76736e4	944.59060	FuelOilRange
5.544	MM	668.51788	59.32564	Surrogate
7.667	HHA+	9743.50488	360.15779	LubeOilRange

Totals: 1364.07403

Results obtained with enhanced integrator!

References

Adriano, D. C., Bollag, J.M., Frankenberger, W. T., Sims, R. C. (2000). Bioremediation of Contaminated Soils. *European Journal of Soil Science* 51(3): 541.

Atlantic Rick Based Corrective Action (RBCA) Guidelines for Laboratories. (1999). Tier 1 and Tier 2 Petroleum Hydrocarbon Methods (1999). Draft Version 1.0.

Alexander, M. (1994). *Biodegradation and Bioremediation.* Academic Press, San Diego.

Alexander, M, (1999). *Biodegradation and Bioremdiation.* Academic Press. San Diego.

Arthurs, P., W.H. Stiver, and R.G. Zytner. (1995). "Passive Volatilization of Gasoline from Soil." *Journal of Soil Contamination.* 4: 123-135.

ASTM. (2002). Standard test method for particle-size analysis of soils.(D422-63). In: *2004 Annual book of ASTM Standards*. Sec. 8, Vol. 4, ASTM, Philadelphia, pp. 10-17.

Atlas, R.M. (1995). *Bioremediation.* Chemical and Engineering News, 73 (14) : 32-42.

Atlas, R.M., Bartha, R. (Eds.). (1998). *Microbial Ecology. Fundamentals and Applications*. Benjamin/Cummings science Publishers, Menlo Park, CA.

ATSDR. (1999). Toxicological profile for total petroleum hydrocarbons (TPH). *Agency for Toxic Substances and Disease Registry, Public Health Service.* U.S. Department of Health and Human Services, Atlanta, GA.

Baker, K.H., Herson, D.S. (1994). *Bioremediation.* McGraw-Hill, New York.

Chang Z.Z., Weaver R.W. (1998). Organic Bulking Agents for enhancing Oil bioremediation in soil. *Journal of Soil Contamination.* 1, 173-180.

Chen, Z., Huang G. H., and Chakma A. (2000). Risk assessment of a petroleum-contaminated site through a multi-phase and multi-component modeling . *Journal of Petroleum Science & Engineering.* 26 {1}273-281.

Cheng, H. H., Mulla, D.J. (1999). *The Soil Environment.* Chapter 1. In Adriano D.C., Bollag, J.M., Frankenberger, Jr., Sims, R.C., {Ed.}, Bioremediation of Contaminated Soils. American Society of Agronomy, Madison, Wisconsin, USA.

Canadian Council of Ministers of the Environment (CCME), (2004). *Petroleum Hydrocarbons - Overview / Rationale* [online]. Updated 11 August 2005 [cited 31 March 2004]. Available from: http://www.ccme.ca/ourwork/standards.html?category_id=6

Cole, G. M. (1994). *Assessment and Remediation of Petroleum Contaminated Sites.* Boca Raton: Lewis Publishers.

Cookson J.T (Ed). (1995). *Bioremediation Engineering*: Design and Application. McGraw Hill.

Crawford R.L., Crawford D.L. (1996). *Bioremediation: Principles and Applications.* Cambridge Univeristy Press. Great Britain.

Cunningham, C.J., Philip, J.C. (2000). "Comparison of Bioaugmentation and Biostimulation in Ex Situ Treatment of Diesel Contaminated Soil." *Land Contamination & Reclamation.* 8(4): 261-270

Custance, S., Ruth, M.P. (1992). Environemental fate of the chemical mixtures: crude oil, JP-5, mineral spirits, and diesel fuel. *Journal of Soil Contamination.* 1(4): 379-386.

Dames and Moore. (1997). *European oil industry guideline for risk-based assessment of contaminated sites.* A report prepared for the CONCAWE Water Quality Management Group by its Special Task Force (WQ/STF-27): CONCAWE Brussels

Demque, D.E. (1994). *"Land Treatment Testing of Diesel Contaminated Soils Using Bioremediation."* M.Sc. Thesis, Royal Military College of Canada, Kingston, Ontario.

Demque, D.E., K.W. Biggar and J.A. Heroux. (1997). *"Land Treatment of Diesel Contaminated soil."* Canadian Geotechnical Journal. 34: 421-431.

Douglas, G.S., McCarthy, K.J., Dahlen, D.T., Seavey, J.A., Steinhauer, W.G., Pince, R.C., Elmendorf, D.L. (1992). The use of hydrocarbon analyses for environmental assessment and remediation. *Journal of Soil Contamination.* 1: 197-216.

Dragun, J. (1998). *The Soil Chemistry of Hazardous Materials.* 2nd Edition. Amherst Scientific Publishers, Amherst, MA.

Edgehill, R. (1992). Factors influencing the success of bioremediation. *Australian Biotechnology.* 2 (5): 297-301.

Environment Canada, (2002). Environment Canada - Ontario Region - Environmental Protection Branch. *TAB #2: Site Assessment Procedures* [online]. Updated 19 November 2002 [cited 31 March 2004]. Site Assessment Process Diagram [gif]. Available from: http://www.on.ec.gc.ca/pollution/ecnpd/tabs/tab02-e.html.

Environment Canada, (2003). Environment Canada - Atlantic Region – Waste Management and Remediation. *Contaminated Sites* [online]. [cited 31 March 2004]. Available from: http://www.atl.ec.gc.ca/epb/wastemgmt/contamsite.html.

Frankenberger, Jr., W.T., K.D. Emerson, and D.W. Turner. (1989). "In Situ Bioremediation of an Underground Diesel Fuel Spill: A Case History." *Environmental Management.* 13(3): 325-332.

Gallego, Jose L.R., J. Loredo, J.F Llamas, F. Vazquez, and J. Sanchez. (2001). "Bioremediation of Diesel-Contaminated Soil: Evaluation of Potential In Situ Techniques by Study of Bacterial Degradation." *Biodegradation.* 12: 325-335.

Gogoi, B.K., N.N. Dutta, P. Goswami, and T.R. Krishna Mohan. (2003). "A Case Study of Bioremediation of Petroleum-Hydrocarbon Contaminated Soil at a Crude Oil Spill Site." *Advances In Environmental Research* 7: 767-782.

Gustafson J. (1997). *Using TPH in risk-based corrective action.* Shell Development Corporation. Published by U.S. Environmental Protection Agency, Office of Underground Storage Tanks.

Heath, J.S., K. Kobis, and S.L. Sayer. (1993). "Review of Chemical, Physical and Toxicology Properties of Components of Total Petroleum Hydrocarbons." *Journal of Soil Contamination.* 2: 221-234.

Hejazi, R.F. (2002). *Oily sludge degradation study under arid conditions using a combination of landfarm and bioreactor techonogies.* Ph.D. Thesis. Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

Heitzer, A.H. and G.S. Sayler. (1993). Monitoring the Efficacy of Bioremediation. *Trends in Biotechnology*. 11: 334-343.

Hillel, D. (1980). *Soil Structure and aggregation.* In: Introduction to Soil Physics. Academic Press, London. 40-52, pp. 200-204.

Husesemann, M.H. (1994). Guildlines for the land-treating petroleum hydrocarbon contaminated soils. *Journal of Soil Contamination*. 3 (3), 299-318.

Jim, N., (1990). Remediation of Petroleum Contaminated Soils. *Journal of Pollution Engineering*. 22, 46-52.

Jorgensen K.S., Puustinen J., and Suortti, A.M. (2000). Bioremediation of petroleum-contaminated soil by composting in biopiles. *Environmental Pollution.* 107: 245-254.

Karen M. (2001). *Introduction to Microbiology*: A laboratory manual for biology 3050: Revised Edition: Department of Biology: Memorial University of Newfoundland

Katherine E. S. (2001). Integrated Energy Statistics Division, Energy Information Administration (EIA), Annual Energy Review. USA.

King, R.B., G.M., Long and J.K. Sheldon. (1992). *Practical environmental Bioremediation*. Lewis Publ., Boca Raton, FL.

Kirchmann,H., and Wasiyhun E. (1998). Biodegradation of petroleum-based oil wastes through composting. *Biodgeradtion.* 9: 151-156.

Kosaric, N. (2001). Biosurfactants and their applications for soil bioremediation. *Food Technology Biotechnology*. 39 (4) 295-304.

Kostecki,P. T., and Calabrese, E. J. (1989). *Petroleum contaminated soils: remediation techniques, environmental fate, risk assessment.* Chelsea, MI: Lewis Publishers.

Langley, A., Gilbey, M., and Kennedy, B. (2003). Determination of Total Petroleum Hydrocarbons in Soil. *Proceedings of the Fifth National Workshop on the Assessment of Site Contamination.* National Environment Protection Council Service Corporation 2003 (NEPC) Adelaide SA.

Loehr, R.C., McMillen, S.J., and Wabster M.T. (2001). "Predictions of biotreatability and actual results: soils with petroleum hydrocarbons". *Practice periodical of hazardous, toxic and radioactive waste management.* 78-87

Lye, L.M. (2003). "Some Applications of Statistical Design of Experiment Methodology in Civil Engineering." *In Proceedings of the Annual Conference of the CSCE*, Moncton, NB. Canada.

Ma, Z. (1998). *Bioremediation of petroleum hydrocarbon contaminated soi using indigenous cultures.* MENG-Thesis. Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

McCrary, G. (1998). *Schematic Diagram of Biodegradation* [online]. [cited 31 March 2004]. Presentation by the Reagents of New Mexico State University. Available from: http://www.swbic.org/education/workshops/microbes/documents/bioremediation.ppt.

Mallawantantri, A.P., McConkey, B..G., and Mulla, D.J. (1996). "Characterization of pesticide sorption and degradation in macropore linings and soil horizons of Thatuna silt loam". *Journal of Environmental Quality.* 25: 227-235.

Marchal R., Penet S., Solano-Serena, F., and Vanadecastleele, J.P. (2003). Gasoline and Diesel Oil Biodegradation". *Oil and Gas Science and Technology*. Rev. IFP, 58(4): 441-448.

Marquez-Rocha, F J., Hernandez-Rodriguèz, V., and Lamella. T. (2001). "Biodegradation of Diesel Oil in Soil by Microbial Consortium". *Water, Air and Soil Pollution*. 128: 313-320

MendozaEspinosa and Stephenson, T. (1996). Grease biodegradation of hydrocarbons: is bioaugmentation more effective than natural populations for start-up? *Water Science and Techonolgy*. 34, 303-308.

Moller, J., Gaarn, H., Steckel, T., Wedebye, E.B., and Westermann P.. (1995). "Inhibitory Effects on Degradation of Diesel Oil in Soil Microcosms by a Commercial Bioagumentation Product." *Bulletin of Environmental Contamination and Toxicology*. 54: 913-918.

Montgomery, D.G. {2001}: *Design and Analysis of Experiments*. 5th Edition, John Wiley and Sons, Inc.

Morgan, P., Lee, S.A., Lewis, S.T., Sheppard, .A.N, and Watkinson, R.J. (1993). Growth and biodegradation by white-rot fungi in soil. *Soil Biology and Biochemistry*. 25, 279-287.

Nadim, F., Hoag, G.E., Liu, S., Carley., R.J. (2000). "Detection & Remediation of soil and aquifer systems contaminated with petroleum products: An overview". *Journal of Petroleum Science and Engineering*. 26, 169-178.

Norris, R.D. (1994). *In situ bioremediation of soils and groundwater contaminated with petroleum hydrocarbons*. p. 17-37. In R.D. Norris et al. (ed). Handbook of bioremediation. Lewis Publ., Boca Raton, FL.

Olsen, S.R., Sommers, L.E. (1982). Phosphorus. *In: Page, A. L. (Ed.), Methods of Soils Analysis Part 2.* Chemical and Microbiological Properties. American Society of Agronomy, Madison, WI, pp. 403-430.

Rahman, K.S.M., Thahira-Rahman, J., Lakshmanaperumalsamy, P., Banat I.M. (2002). "Towards effiecient crude oil degradation by a mixed bacterial consortium". *Bioresource Technology.* 85: 257-261.

Rhykerd, R.L., B. Crews, K.J. Mclnnes, and R.W. Weaver. (1999). "Impact of Bulking Agents, Forced Aeration and Tillage on Remediation of Oil-Contaminated Soil." *Bioresource Technology.* 67: 279-285.

Riser-Roberts, E. (1998). *Remediation of Petroleum Contaminated Soils, Biological, Physical and Chemical Processes*. Lewis Publishers, Boca Raton.

Rosenbaum-Wilkinson, C. (1994). *Hydrogeologic information and groundwater modelling.* Chapter 3. In K.H. Baker and D.S. Herson (ed). Bioremediation. McGraw-Hill, New York.

Russell, D.L (1992). *Remediation Maual for petroleum-Contamianted Sites*. Technomic Publishing Company, Inc. Pennsylvania, USA.

Sheldrick, B.H. (1984). *Analytical methods manual.* Research Branch Agriculture Canada.

Strbak .L. (2000). *In Situ Flushing with Surfactants and Cosolvents.* US. Environmental Protection Agency. Office of Solid Waste and Emergency Response. Technology Innovation Office, Washington, DC.

Suthersan, S.S. (1997). *Remediation Engineering:* Design Concepts. CRC Press, Boca Raton.

Tecator Co. (1996). *Soxtec system HT2 Instruction Manual.* Rev. 2.0 Part no. 100 2296. Tecator AB, Sweden.

Thomas, J. M., and C.H., Ward. (1993). *Introduced organisms for subsurface bioremediation.* pp. 227-244. In Norris et al. (Ed). Handbook of bioremediation. Lewis Publ., Boca Raton, FL.

Troy, M.A., (1994). *Bioengineering of soils and groundwaters.* P. 173-201. In K.H Baker and D.S. Herson (ed). Bioremediation. McGraw-Hill, New York.

US Environmental Protection Agency. (1986b). EPA Method 3541. *Automated Soxhlet Extraction.* Test Methods for Evaluating Solid Wastes. 3rd Ed.Update. SW-846.

US Environmental Protection Agency. (1986) Method 3600C. *Clean up.* Test Methods for Evaluating Solid Wastes. 3rd Ed.Update. SW-846.

US Environmental Protection Agency. (1986). EPA Method 7140. *Calcium (Atomic Absorption, Direct Aspiration).* Test Methods for Evaluating Solid Wastes. 3rd Ed.Update. SW-846.

US Environmental Protection Agency. (1986). EPA Method 7380. *Iron (Atomic Absorption, Direct Aspiration).* Test Methods for Evaluating Solid Wastes. 3rd Ed.Update. SW-846.

US Environmental Protection Agency. (1986). EPA Method 7450. *Magnessium (Atomic Absorption, Direct Aspiration).* Test Methods for Evaluating Solid Wastes. 3rd Ed.Update. SW-846.

US Environmental Protection Agency. (1986). EPA Method 7610. *Potasium (Atomic Absorption, Direct Aspiration).* Test Methods for Evaluating Solid Wastes. 3rd Ed.Update. SW-846.

US Environmental Protection Agency. (1986a). EPA Method 8015. *Nonhalogenated Organics Using GC/FID.* Test Methods for Evaluating Solid Wastes. 3rd Ed.Update. SW-846.

US Environmental Protection Agency. (1999). *Use of Monitored Natural attenuation at superfund, RCRA Corrective Action and Underground Storage Tank Sites.* OSWER Directive Number 9200.4-17P, Office of Solid Waste and Emergency Response, Washington, D.C.

US Environmental Protection Agency. (2004). EPA 510-R-04-002. *How to Evaluate Alternatives Cleanup Technologies for Underground Storage Tank Sites. A Guide for Corrective Action Plan Reviewers.* Office of Solid Waste and Emergency Response, Washington, D.C.

Vasudevan, N., and Rajaram, P. (2001). Bioremediation of oil sludge-contaminated soil. *Environmental International.* 26: 409-411.

Vidali, M. (2001). *Bioremediation: An Overview.* Pure Applied Chemistry.,73 (7): 1163-1172.

Walworth, J.L., Woolard, C.R., Braddock, J.F. and Reynolds, C.M. (1997). "The role of soil nitrogen concentration in Bioremediation". *Fourth International In Situ and On-Site Bioremediation Symposium,* New Orleans, April 28-May 1, Battelle – Bioremediation, 4 (4), 283-288.

Williams, C.M., Grimes, J.L., and Mikkelsen, R.L. (1999). "The use of poultry litter as a co-substate and source of inorganic nutrients and microorganisms for the ex situ biodegradation of petroleum compounds". *Poultry Science.* 78: 956-964

Yong, R.N., Mulligan C.N. (2004) *Natural attenuation of contaminants in soils.* Boca Raton, FL : Lewis Publishers.

Zytner, R.G., Salb, A., Brook, T.R., Leunissen, M., and Stiver, W.H. (2001). "Bioremediation of Diesel Fuel Contaminated Soil." *Canadian Journal of Civil Engineering.* 28(1): 131-140.